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(54) Title: DNA ENCODING HUMAN ALPHA 1 ADRENERGIC RECEPTORS AND USES THEREOF (57) Abstract his invention provides an isolated nucleic acid, vectors, transformed mammalian cells and non-human transgenic animals that encode and express normal or mutant alpha 1a, alpha 1b and alpha 1c adrenergic receptor genes. This invention also provides a protein, antibody directed to the protein and pharmaceutical compounds related to alpha 1a, alpha 1b and alpha 1c adrenergic receptors. This invention provides nucleic acid probe, antisense oligonucleotide complementary to alpha 1a, alpha 1b and alpha 1c adrenergic receptor genes. This invention further provides methods for determining ligand binding, detecting expression, drug screening, and treatments for alleviating abnormalities associated with human alpha 1a, alpha 1b and alpha 1c adrenergic receptors.		

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DNA ENCODING HUMAN ALPHA 1 ADRENERGIC RECEPTORS AND
USES THEREOF

Background of the Invention

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Throughout this application various publications are referred to by partial citations within parenthesis. Full citations for these publications may be found at the end of the specification immediately preceding the
10 claims. The disclosures of these publications, in their entireties, are hereby incorporated by reference into this application in order to more fully describe the state of the art to which this invention pertains.

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Although adrenergic receptors (ARs) bind the same endogenous catecholamines (epinephrine and norepinephrine, NE) their physiological as well as pharmacological specificity is markedly diverse. This diversity is due primarily to the existence of at least
20 nine different proteins encoding three distinct adrenergic receptors types (α_1 , α_2 , and β). These proteins belong to the super-family of G-protein coupled receptors, and are characterized by a single polypeptide chain which span the plasma membrane seven
25 times, with an extracellular amino terminus, and a cytoplasmic carboxyl terminus. The molecular cloning of three genes encoding α_1 -ARs supports the existence of pharmacologically and anatomically distinct α_1 -receptor subtypes. The α_{1b} -receptor was originally
30 cloned from a hamster smooth muscle cell line cDNA library, and encodes a 515 a.a. peptide that shows 42-47% homology with other ARs. The message for the α_{1b} -receptor is abundant in rat liver, heart, cerebral cortex and kidney, and its gene was localized to human
35 chromosome 5 (4). A second cDNA clone from a bovine brain library was found which encoded a 466-residue polypeptide with 72% homology to the α_{1b} -AR gene. It was further distinguished from α_{1b} by the finding that its expression was restricted to human hippocampus, and

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by its localization to human chromosome 8 and it has been designated as the α_{1c} -AR (20). The cloning of an α_{1a} -AR has been reported recently. This gene, isolated from a rat brain cDNA library, encodes a 560-residue polypeptide that shows 73% homology with the hamster α_{1b} -receptor. The message for this subtype is abundant in rat vas deferens, aorta, cerebral cortex and hippocampus, and its gene has been localized to human chromosome 5 (12).

Pharmacological studies have demonstrated the existence of two α_1 -adrenergic receptor subtypes. The studies of α_1 -AR-mediated responses in vascular tissue suggested the possible existence of receptor subtypes, based on the potency and efficacy of adrenergic agonists, as well as differential sensitivity of α_1 receptor-mediated responses to extracellular calcium and calcium channel blockers (6, 24). Although radioligand binding studies of brain α_1 -ARs with either [3 H]WB4101 and [3 H]prazosin showed good agreement with the potency of α -adrenergic antagonists on vascular responses (23, 10), subsequent binding studies of rat brain α_1 -ARs provided strong evidence for the existence of receptor heterogeneity, based on the relative affinities for prazosin and WB4101 (15). These observations were supported by the finding that chloroethylclonidine (CEC) inactivated 50% of the α_1 sites from rat cerebral cortex and 80% of the binding sites from liver or spleen (α_{1b}), but did not inactivate α_1 -receptors from the hippocampus or vas deferens (α_{1a}) (14). Taken together, these results suggested a classification of the α_{1a} -subtype as high affinity for WB4101 and insensitive to alkylation by CEC, and α_{1b} -subtype as 10 to 20 fold lower affinity for WB4101, but sensitive to inactivation by CEC. Consistent with this evidence the transfection of the hamster α_{1b} gene into COS-7 cells induced the expression of an α_1 -receptor with high

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affinity for WB4101, 95% of which could be inactivated by CEC. Conversely, upon expression of the rat α_{1a} receptor gene in COS-7 cells, it showed a 10-fold higher affinity for WB4101 than the α_{1b} -receptor, and the binding site was resistant to inactivation by CEC.

The existence of the α_{1c} receptor was not predicted from pharmacological data and upon expression it showed 16 and 30 fold higher affinity for WB4101 and phentolamine respectively, than the α_{1b} -receptor and was partially inactivated (65%) by CEC.

Molecular cloning and pharmacological studies have demonstrated the existence of at least three α_1 -adrenergic receptor subtypes. However, it is not clear whether the pharmacological properties of these three cognates might be due also to species differences. This caveat is particularly relevant in the case of the bovine α_{1c} receptor, due to its restricted species and tissue expression. The cloning and expression of the human α_1 adrenergic receptors will allow the further characterization of the pharmacology of the individual human α_1 receptor subtypes.

Summary of the Invention

This invention provides and isolated nucleic acid molecule encoding a human α_1 adrenergic receptor.

5 This invention further provides an isolated nucleic acid molecule encoding a human α_{1a} receptor. In one embodiment of this invention, the nucleic acid molecule comprises a plasmid pcEXV- α_{1a} . This invention also provides an isolated nucleic acid molecule encoding a
10 human α_{1b} receptor. In one embodiment of this invention, the nucleic acid molecule comprises a plasmid pcEXV- α_{1b} . This invention further provides an isolated nucleic acid molecule encoding a human α_{1c} receptor. In one embodiment of this invention, the
15 nucleic acid molecule comprises a plasmid pcEXV- α_{1c} .

This invention also provides vectors such as plasmids comprising a DNA molecule encoding a human α_{1a} receptor, adapted for expression in a bacterial, a yeast cell, or
20 a mammalian cell which additionally comprise regulatory elements necessary for expression of the DNA in the bacteria, yeast or mammalian cells so located relative to the DNA encoding the human α_{1a} receptor as to permit expression thereof. This invention also provides
25 vectors such as plasmids comprising a DNA molecule encoding a human α_{1b} receptor, adapted for expression in a bacterial, a yeast cell, or a mammalian cell which additionally comprise regulatory elements necessary for expression of the DNA in the bacteria, yeast or
30 mammalian cells so located relative to the DNA encoding the human α_{1b} receptor as to permit expression thereof. This invention also provides vectors such as plasmids comprising a DNA molecule encoding a human α_{1c} receptor, adapted for expression in a bacterial, a yeast cell, or
35 a mammalian cell which additionally comprise regulatory elements necessary for expression of the DNA in the bacteria, yeast or mammalian cells so located relative

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to the DNA encoding the human α_{1c} receptor as to permit expression thereof.

5 This invention provides a mammalian cell comprising a DNA molecule encoding a human α_{1a} receptor. This invention also provides a mammalian cell comprising a DNA molecule encoding a human α_{1b} receptor. This invention also provides a mammalian cell comprising a
10 DNA molecule encoding a human α_{1c} receptor.

This invention provides a nucleic acid probe comprising a nucleic acid molecule of at least 15 nucleotides capable of specifically hybridizing with a sequence
15 included within the sequence of a nucleic acid molecule encoding a human α_{1a} receptor. This invention provides a nucleic acid probe comprising a nucleic acid molecule of at least 15 nucleotides capable of specifically hybridizing with a sequence included within the
20 sequence of a nucleic acid molecule encoding a human α_{1b} receptor. This invention provides a nucleic acid probe comprising a nucleic acid molecule of at least 15 nucleotides capable of specifically hybridizing with a sequence included within the sequence of a nucleic acid
25 molecule encoding a human α_{1c} receptor.

This invention provides an antisense oligonucleotide having a sequence capable of specifically binding to any sequences of an mRNA molecule encoding a human α_{1a}
30 receptor so as to prevent translation of the mRNA molecule. This invention provides an antisense oligonucleotide having a sequence capable of specifically binding to any sequences of an mRNA molecule encoding a human α_{1b} receptor so as to prevent
35 translation of the mRNA molecule. This invention provides an antisense oligonucleotide having a sequence capable of specifically binding to any sequences of an

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mRNA molecule encoding a human α_1 receptor so as to prevent translation of the mRNA molecule.

5 This invention provides method for detecting expression
of a specific human α_1 adrenergic receptor, which
comprises obtaining RNA from cells or tissue,
contacting the RNA so obtained with a nucleic acid
probe comprising a nucleic acid molecule of at least 15
10 nucleotides capable of specifically hybridizing with a
sequence included within the sequence of a nucleic acid
molecule encoding a human α_1 receptor under hybridizing
conditions, detecting the presence of any mRNA
hybridized to the probe, the presence of mRNA
15 hybridized to the probe indicating expression of the
specific human α_1 adrenergic receptor, and thereby
detecting the expression of the specific human α_1
adrenergic receptor.

20 This invention provides a method for detecting the
expression of a specific human α_1 adrenergic receptor
in a cell or tissue by in situ hybridization which
comprises, contacting the cell or tissue with a nucleic
acid probe comprising a nucleic acid molecule of at
least 15 nucleotides capable of specifically
25 hybridizing with a sequence included within the
sequence of a nucleic acid molecule encoding a human α_1
receptor under hybridizing conditions, detecting the
presence of any mRNA hybridized to the probe, the
presence of mRNA hybridized to the probe indicating
30 expression of the specific human α_1 adrenergic
receptor, and thereby detecting the expression of the
specific human α_1 adrenergic receptor.

35 This invention provides a method for isolating a
nucleic acid molecule encoding a receptor by nucleic
acid sequence homology using a nucleic acid probe, the
sequence of which is derived from the nucleic acid

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sequence encoding a human α_1 adrenergic receptor.

5 This invention provides a method for isolating a nucleic acid molecule encoding a human α_1 adrenergic receptor which comprises the use of the polymerase chain reaction and oligonucleotide primers, the sequence of which are derived from the nucleic acid sequence encoding a human α_1 adrenergic receptor.

10 This invention provides a method for isolating a human α_1 adrenergic receptor protein which comprises inducing cells to express the human α_1 adrenergic receptor protein, recovering the human α_1 adrenergic receptor from the resulting cells, and purifying the human α_1 adrenergic receptor so recovered.

20 This invention provides an antibody to the human α_{1a} adrenergic receptor. This invention also provides an antibody to the human α_{1b} adrenergic receptor. This invention also provides an antibody to the human α_{1c} adrenergic receptor.

25 A pharmaceutical composition comprising an amount of a substance effective to alleviate the abnormalities resulting from overexpression of a human α_{1a} adrenergic receptor and a pharmaceutically acceptable carrier is provided by this invention. A pharmaceutical composition comprising an amount of a substance effective to alleviate the abnormalities resulting from overexpression of a human α_{1b} adrenergic receptor and a pharmaceutically acceptable carrier is provided by this invention. A pharmaceutical composition comprising an amount of a substance effective to alleviate the abnormalities resulting from overexpression of a human α_{1c} adrenergic receptor and a pharmaceutically acceptable carrier is provided by this invention.

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A pharmaceutical composition comprising an amount of a substance effective to alleviate abnormalities resulting from underexpression of a human α_{1a} adrenergic receptor and a pharmaceutically acceptable carrier is provided by this invention. A pharmaceutical composition comprising an amount of a substance effective to alleviate abnormalities resulting from underexpression of a human α_{1b} adrenergic receptor and a pharmaceutically acceptable carrier is provided by this invention. A pharmaceutical composition comprising an amount of a substance effective to alleviate abnormalities resulting from underexpression of a human α_{1c} adrenergic receptor and a pharmaceutically acceptable carrier is provided by this invention.

This invention provides a transgenic non-human mammal whose genome comprises a nucleic acid molecule encoding a human α_1 adrenergic receptor, the DNA molecule so placed as to be transcribed into antisense mRNA complementary to mRNA encoding a human α_1 adrenergic receptor and which hybridizes to mRNA encoding a human α_1 adrenergic receptor thereby reducing its translation.

This invention provides a method for determining the physiological effects of varying the levels of expression of a specific human α_1 adrenergic receptor which comprises producing a transgenic non-human mammal whose levels of expression of a human α_1 adrenergic receptor can be varied by use of an inducible promoter.

This invention provides method for determining the physiological effects of expressing varying levels of a specific human α_1 adrenergic receptor which comprises producing a panel of transgenic non-human mammals each

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expressing a different amount of the human α_1 adrenergic receptor.

5 This invention provides a method for determining whether a ligand not known to be capable of specifically binding to a human α_1 adrenergic receptor can bind to a human α_1 adrenergic receptor, which comprises contacting a mammalian cell comprising a plasmid adapted for expression in a mammalian cell
10 which further comprises a DNA molecule which expresses a human α_1 adrenergic receptor on the cell surface with the ligand under conditions permitting binding of ligands known to bind to a human α_1 adrenergic receptor, detecting the presence of any ligand bound to
15 the human α_1 adrenergic receptor, the presence of bound ligand thereby determining that the ligand binds to the human α_1 adrenergic receptor.

20 This invention provides a method for screening drugs to identify drugs which interact with, and specifically bind to, a human α_1 adrenergic receptor on the surface of a cell, which comprises contacting a mammalian cell which comprises a plasmid adapted for expression in a mammalian cell which further comprises a DNA molecule
25 which expresses a human α_1 adrenergic receptor on the cell surface with a plurality of drugs, determining those drugs which bind to the human α_1 adrenergic receptor expressed on the cell surface of the mammalian cell, and thereby identifying drugs which interact
30 with, and bind to, the human α_1 adrenergic receptor.

35 This invention provides a method for identifying a ligand which binds to and activates or blocks the activation of, a human α_1 adrenergic receptor expressed on the surface of a cell, which comprises contacting a mammalian cell which comprises a plasmid adapted for expression in a mammalian cell which further comprises

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a DNA molecule which expresses a human α_1 adrenergic receptor on the cell surface with the ligand, determining whether the ligand binds to and activates or blocks the activation of the receptor using a bioassay such as a second messenger assays.

This invention also provides a method for identifying a ligand which is capable of binding to and activating or inhibiting a human α_1 adrenergic receptor, which comprises contacting a mammalian cell, wherein the membrane lipids have been labelled by prior incubation with a labelled lipid precursor molecule, the mammalian cell comprising a plasmid adapted for expression in a mammalian cell which further comprises a DNA molecule which expresses a human α_1 adrenergic receptor with the ligand and identifying an inositol phosphate metabolite released from the membrane lipid as a result of ligand binding to and activating an α_1 adrenergic receptor.

This invention also provides a method for identifying a ligand that is capable of binding to and activating or inhibiting a human α_1 adrenergic receptor, wherein the binding of ligand to the adrenergic receptor results in a physiological response, which comprises contacting a mammalian cell which comprises a plasmid adapted for expression in a mammalian cell which further comprises a DNA molecule which expresses a human α_1 adrenergic receptor with a calcium sensitive fluorescent indicator, removing the indicator that has not been taken up by the cell, contacting the cells with the ligand and identifying an increase or decrease in intracellular Ca^{+2} as a result of ligand binding to and activating or inhibiting α_1 adrenergic receptor activity.

This invention provides a method for detecting the

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- presence of a human α_{1a} adrenergic receptor on the surface of a cell, which comprises contacting the cell with an antibody to human α_{1a} adrenergic receptor under conditions which permit binding of the antibody to the receptor, detecting the presence of any of the antibody bound to the human α_{1a} adrenergic receptor and thereby the presence of a human α_{1a} adrenergic receptor on the surface of the cell.
- 10 This invention provides a method for detecting the presence of a human α_{1b} adrenergic receptor on the surface of a cell, which comprises contacting the cell with an antibody to human α_{1b} adrenergic receptor under conditions which permit binding of the antibody to the receptor, detecting the presence of any of the antibody bound to the human α_{1b} adrenergic receptor and thereby the presence of a human α_{1b} adrenergic receptor on the surface of the cell.
- 20 This invention provides a method for detecting the presence of a human α_{1c} adrenergic receptor on the surface of a cell, which comprises contacting the cell with an antibody to human α_{1c} adrenergic receptor under conditions which permit binding of the antibody to the receptor, detecting the presence of any of the antibody bound to the human α_{1c} adrenergic receptor and thereby the presence of a human α_{1c} adrenergic receptor on the surface of the cell.
- 30 This invention provides a method of treating an abnormal condition related to an excess of activity of a human α_1 adrenergic receptor subtype, which comprises administering an amount of a pharmaceutical composition effective to reduce α_1 adrenergic activity as a result of naturally occurring substrate binding to and activating a specific α_1 adrenergic receptor.

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5 This invention provides a method for treating abnormalities which are alleviated by an increase in the activity of a specific human α_1 adrenergic receptor, which comprises administering a patient an amount of a pharmaceutical composition effective to increase the activity of the specific human α_1 adrenergic receptor thereby alleviating abnormalities resulting from abnormally low receptor activity.

10 This invention provides a method for diagnosing a disorder or a predisposition to a disorder associated with the expression of a specific human α_1 adrenergic receptor allele which comprises: a.) obtaining DNA from subjects suffering from a disorder; b.) performing a
15 restriction digest of the DNA with a panel of restriction enzymes; c.) electrophoretically separating the resulting DNA fragments on a sizing gel; d.) contacting the gel with a nucleic acid probe labelled with a detectable marker and which hybridizes to the
20 nucleic acid encoding a specific human α_1 adrenergic receptor; e.) detecting the labelled bands which have hybridized to the DNA encoding the specific α_1 adrenergic receptor labelled with the detectable marker to create a unique band pattern specific to the DNA of
25 subjects suffering with the disorder; f.) preparing DNA for diagnosis by steps a- e; g.) comparing the unique band pattern specific to the DNA of patients suffering from the disorder from step e and DNA obtained for diagnosis from step f to determine whether the patterns
30 are the same or different and to diagnose thereby predisposition to the disorder if the patterns are the same.

35 This invention provides a method for identifying a substance capable of alleviating the abnormalities resulting from overexpression of a specific human α_1 adrenergic receptor which comprises administering a

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substance to the transgenic non-human mammal comprising the DNA encoding a specific α_1 adrenergic receptor and determining whether the substance alleviates the physical and behavioral abnormalities displayed by the transgenic nonhuman mammal as a result of overexpression of the human α_1 adrenergic receptor subtype.

This invention provides a method for identifying a substance capable of alleviating the abnormalities resulting from underexpression of a human α_1 adrenergic receptor subtype, which comprises administering a substance to a non-human transgenic mammal which is expressing a human α_1 adrenergic receptor incapable of receptor activity or is underexpressing the human α_1 adrenergic receptor subtype, and determining whether the substance alleviates the physical and behavioral abnormalities displayed by the transgenic nonhuman mammal as a result of underexpression of a human α_1 adrenergic receptor subtype.

This invention provides a method of treating abnormalities in a subject, wherein the abnormality is alleviated by the reduced expression of a human α_1 adrenergic receptor subtype which comprises administering to a subject an effective amount of the pharmaceutical composition effective to reduce expression of a specific α_1 adrenergic receptor subtype.

This invention provides a method of treating abnormalities resulting from underexpression of a human α_1 adrenergic receptor which comprises administering to a subject an amount of a pharmaceutical composition effective to alleviate abnormalities resulting from underexpression of the specific human α_1 adrenergic receptor.

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Brief Description of the Figures

Figures 1A-I. Nucleotide Sequence and Deduced Amino Acid Sequence of Novel Human Alpha-1a Adrenergic Receptor.

5 Nucleotides are presented in the 5' to 3' orientation and the coding region is numbered starting from the initiating methionine and ending in the termination codon. Deduced amino acid sequence by translation of
10 a long open reading frame is shown, along with the 5' and 3' untranslated regions. Numbers in the left and right margins represent nucleotide (top line) and amino acid (bottom line) numberings, starting with the first position as the adenosine (A) and the initiating
15 methionine (M), respectively.

Figures 2A-H. Nucleotide Sequence and Deduced Amino Acid Sequence of Novel Human Alpha-1b Adrenergic Receptor. Nucleotides are presented in the 5' to 3' orientation and the coding region is numbered starting from the initiating methionine and ending in the termination codon. Deduced amino acid sequence by translation of a long open reading frame is shown, along with the 5' and 3' untranslated regions. Numbers
20 in the left and right margins represent nucleotide (top line) and amino acid (bottom line) numberings, starting with the first position as the adenosine (A) and the initiating methionine (M), respectively.

Figures 3A-G. Nucleotide Sequence and Deduced Amino Acid Sequence of Novel Human Alpha-1c Adrenergic Receptor.

30 Nucleotides are presented in the 5' to 3' orientation and the coding region is numbered starting from the initiating methionine and ending in the termination codon. Deduced amino acid sequence
35 by translation of a long open reading frame is shown,

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along with the 5' and 3' untranslated regions. Numbers in the left and right margins represent nucleotide (top line) and amino acid (bottom line) numberings, starting with the first position as the adenosine (A) and the initiating methionine (M), respectively.

Figures 4A-D. Alignment of the Human Alpha-1a, H318/3 Alpha-1a, and Rat Alpha-1a Adrenergic Receptors.

The deduced amino acid sequence of the human α_{1a} receptor (first line), from the starting methionine (M) to the stop codon (*), is aligned with the previously published human " α_{1a} " adrenergic receptor clone, H318/3 (2)(second line) and with the rat alpha-1a (12)(third line). Also shown is a consensus amino acid sequence (fourth line), containing a hyphen at a particular position, when all receptors have the same amino acid or an amino acid at this position, when there is disparity in the three receptors. Dots indicate spaces corresponding to no amino acid at this position. Note that the human and rat α_{1a} receptors have greater homology in the amino (positions 1-90) and carboxyl (positions 440-598) termini than do the previously published " α_{1a} " (H318/3) and rat α_{1a} receptors (see text). Dots indicate spaces corresponding to no amino acid at this position. Numbers above amino acid sequences correspond to amino acid positions, starting with the initiating methionine (M) and ending with the termination codon (*).

Figures 5A-D. Alignment of the Human Alpha-1b, Hamster Alpha-1b, and Rat Alpha-1b Adrenergic Receptors.

The deduced amino acid sequence of the human α_{1b} receptor (third line), from the starting methionine (M) to the stop codon (*), is aligned with the previously published rat α_{1b} adrenergic receptor clone (25)(first line) and with the hamster alpha-1b (4)(second line). Also shown is a consensus amino acid sequence (fourth

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line), containing a hyphen at a particular position, when all receptors have the same amino acid or an amino acid at this position, when there is disparity in the three receptors. Dots indicate spaces corresponding to no amino acid at this position. Numbers above amino acid sequences correspond to amino acid position, starting with the initiating methionine (M) and ending with the termination codon (*).

Figures 6A-C. Alignment of the Human Alpha-1c and Bovine Alpha-1c Adrenergic Receptors.

The deduced amino acid sequence of the human α_{1c} receptor (first line), from the starting methionine (M) to the stop codon (*), is aligned with the previously published bovine α_{1b} adrenergic receptor clone (13) (first line). Also shown is a consensus amino acid sequence (third line), containing a hyphen at a particular position, when all receptors have the same amino acid or an amino acid at this position, when there is disparity in the three receptors. Dots indicate spaces corresponding to no amino acid at this position. Numbers above amino acid sequences correspond to amino acid position, starting with the initiating methionine (M) and ending with the termination codon (*).

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Figure 7. Illustrates the correlation of inhibition constants (pK_i) for a series of α_1 antagonists at the cloned human α_{1A} , α_{1B} , and α_{1C} receptors with efficiency of blocking contraction of human prostate tissue (pA_2).

Detailed Description of the Invention

This invention provides an isolated nucleic acid molecule encoding a human α_1 adrenergic receptor. This invention also provides an isolated nucleic acid molecule encoding a human α_{1a} adrenergic receptor. This invention also provides an isolated nucleic acid molecule encoding a human α_{1b} adrenergic receptor. This invention also provides an isolated nucleic acid molecule encoding a human α_{1c} adrenergic receptor. As used herein, the term "isolated nucleic acid molecule" means a non-naturally occurring nucleic acid molecule that is, a molecule in a form which does not occur in nature. Examples of such an isolated nucleic acid molecule are an RNA, cDNA, or an isolated genomic DNA molecule encoding a human α_{1a} , human α_{1b} or human α_{1c} adrenergic receptor. As used herein, the term " α_{1a} receptor", " α_{1b} receptor", or " α_{1c} receptor" means a molecule which is a distinct member of a class of α_1 adrenergic receptor molecules which under physiologic conditions, is substantially specific for the catecholamines epinephrine and norepinephrine, is saturable, and having high affinity for the catecholamines epinephrine and norepinephrine. The term " α_1 adrenergic receptor subtype" refers to a distinct member of the class of human α_1 adrenergic receptors, which may be any one of the human α_{1a} , α_{1b} or α_{1c} adrenergic receptors. The term "specific α_1 adrenergic receptor" refers to a distinct member of the group or class of human α_1 adrenergic receptors, which may be any one of the human α_{1a} , α_{1b} or α_{1c} adrenergic receptors. One embodiment of this invention is an isolated human nucleic acid molecule encoding a human α_{1a} adrenergic receptor. Such a molecule may have coding sequences substantially the same as the coding sequence in Figures 1A-1I. The DNA molecule of Figures 1A-1I encodes the sequence of the human α_{1a} adrenergic

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receptor. Another, preferred embodiment is an isolated human nucleic acid molecule encoding a human α_{1b} adrenergic receptor. Such a molecule may have coding sequences substantially the same as the coding sequence in Figures 2A-2H. The DNA molecule of Figures 2A-2H encodes the sequence of the human α_{1b} adrenergic receptor. Another, preferred embodiment is an isolated human nucleic acid molecule encoding a human α_{1c} adrenergic receptor. Such a molecule may have coding sequences substantially the same as the coding sequence in Figures 3A-3G. The DNA molecule of Figures 3A-3G encodes the sequence of the human α_{1c} adrenergic receptor. One means of isolating a nucleic acid molecule encoding a α_1 adrenergic receptor is to screen a genomic DNA or cDNA library with a natural or artificially designed DNA probe, using methods well known in the art. In the preferred embodiment of this invention, α_1 adrenergic receptors include the human α_{1a} , human α_{1b} and human α_{1c} adrenergic receptors and the nucleic acid molecules encoding them were isolated by screening a human genomic DNA library and by further screening of a human cDNA library to obtain the sequence of the entire human α_{1a} , human α_{1b} or human α_{1c} adrenergic receptor. To obtain a single nucleic acid molecule encoding the entire human α_{1a} , α_{1b} or α_{1c} adrenergic receptor two or more DNA clones encoding portions of the same receptor were digested with DNA restriction endonucleases and ligated together with DNA ligase in the proper orientation using techniques known to one of skill in the art. DNA or cDNA molecules which encode a human α_{1a} , α_{1b} or α_{1c} adrenergic receptor are used to obtain complementary genomic DNA, cDNA or RNA from human, mammalian or other animal sources, or to isolate related cDNA or genomic DNA clones by the screening of cDNA or genomic DNA libraries, by methods described in more detail below. Transcriptional regulatory elements from the 5' untranslated region of

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the isolated clone, and other stability, processing, transcription, translation, and tissue specificity determining regions from the 3' and 5' untranslated regions of the isolated gene are thereby obtained.

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This invention provides an isolated nucleic acid molecule which has been so mutated as to be incapable of encoding a molecule having normal human α_1 adrenergic receptor activity, and not expressing native human α_1 adrenergic receptor. An example of a mutated nucleic acid molecule provided by this invention is an isolated nucleic acid molecule which has an in-frame stop codon inserted into the coding sequence such that the transcribed RNA is not translated into protein.

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This invention provides a cDNA molecule encoding a human α_{1a} adrenergic receptor, wherein the cDNA molecule has a coding sequence substantially the same as the coding sequence shown in Figures 1A-1I. This invention also provides a cDNA molecule encoding a human α_{1b} adrenergic receptor, wherein the cDNA molecule has a coding sequence substantially the same as the coding sequence shown in Figures 2A-2H. This invention also provides a cDNA molecule encoding a human α_{1c} adrenergic receptor, wherein the cDNA molecule has a coding sequence substantially the same as the coding sequence shown in Figures 3A-3G. These molecules and their equivalents were obtained by the means further described below.

30

This invention provides an isolated protein which is a human α_1 adrenergic receptor. In one embodiment of this invention, the protein is a human α_{1a} adrenergic receptor having an amino acid sequence substantially similar to the amino acid sequence shown in Figures 1A-1H. In another embodiment of this invention, the protein is a human α_{1b} adrenergic receptor having an

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amino acid sequence substantially similar to the amino acid sequence shown in Figures 2A-2H. In another embodiment of this invention, the protein is a human α_{1c} adrenergic receptor having an amino acid sequence substantially similar to the amino acid sequence shown in Figures 3A-3G. As used herein, the term "isolated protein" is intended to encompass a protein molecule free of other cellular components. One means for obtaining an isolated human α_1 adrenergic receptor is to express DNA encoding the α_1 adrenergic receptor in a suitable host, such as a bacterial, yeast, or mammalian cell, using methods well known to those skilled in the art, and recovering the human α_1 adrenergic receptor after it has been expressed in such a host, again using methods well known in the art. The human α_1 adrenergic receptor may also be isolated from cells which express it, in particular from cells which have been transfected with the expression vectors described below in more detail.

This invention also provides a vector comprising an isolated nucleic acid molecule such as DNA, RNA, or cDNA, encoding a human α_{1a} receptor. This invention also provides a vector comprising an isolated nucleic acid molecule such as DNA, RNA, or cDNA, encoding a human human α_{1b} adrenergic receptor. This invention also provides a vector comprising an isolated nucleic acid molecule such as DNA, RNA, or cDNA, encoding a human α_{1c} adrenergic receptor. Examples of vectors are viruses such as bacteriophages (such as phage lambda), cosmids, plasmids (such as pUC18, available from Pharmacia, Piscataway, NJ), and other recombination vectors. Nucleic acid molecules are inserted into vector genomes by methods well known to those skilled in the art. Examples of such plasmids are plasmids comprising cDNA having a coding sequence substantially the same as: the coding sequence shown in Figures 1A-

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1I, 2A-2H, and 3A-3G. Alternatively, to obtain these vectors, insert and vector DNA can both be exposed to a restriction enzyme to create complementary ends on both molecules which base pair with each other and are then ligated together with a ligase. Alternatively, linkers can be ligated to the insert DNA which correspond to a restriction site in the vector DNA, which is then digested with the restriction enzyme which cuts at that site. Other means are also available.

This invention also provides vectors comprising a DNA molecule encoding a human α_{1a} , vectors comprising a DNA molecule encoding a human α_{1b} adrenergic receptor and vectors comprising a DNA molecule encoding a human α_{1c} adrenergic receptor adapted for expression in a bacterial cell, a yeast cell, or a mammalian cell which additionally comprise the regulatory elements necessary for expression of the DNA in the bacterial, yeast, or mammalian cells so located relative to the DNA encoding a human α_1 adrenergic receptor as to permit expression thereof. DNA having coding sequences substantially the same as the coding sequence shown in Figures 1A-1I may be inserted into the vectors to express a human α_{1a} adrenergic receptor. DNA having coding sequences substantially the same as the coding sequence shown in Figures 2A-2H may be inserted into the vectors to express a human α_{1b} adrenergic receptor. DNA having coding sequences substantially the same as the coding sequence shown in Figures 3A-3G may be inserted into the vectors to express a human α_{1c} adrenergic receptor. Regulatory elements required for expression include promoter sequences to bind RNA polymerase and transcription initiation sequences for ribosome binding. For example, a bacterial expression vector includes a promoter such as the lac promoter and for transcription initiation the Shine-Dalgarno sequence

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and the start codon AUG (Maniatis, et al., Molecular Cloning, Cold Spring Harbor Laboratory, 1982). Similarly, a eukaryotic expression vector includes a heterologous or homologous promoter for RNA polymerase II, a downstream polyadenylation signal, the start codon AUG, and a termination codon for detachment of the ribosome. Such vectors may be obtained commercially or assembled from the sequences described by methods well known in the art, for example the methods described above for constructing vectors in general. Expression vectors are useful to produce cells that express a human α_1 adrenergic receptor. Certain uses for such cells are described in more detail below.

In one embodiment of this invention a plasmid is adapted for expression in a bacterial, yeast, or, in particular, a mammalian cell wherein the plasmid comprises a DNA molecule encoding a human α_{1a} adrenergic receptor, a DNA molecule encoding a human α_{1b} adrenergic receptor or a DNA molecule encoding a human α_{1c} adrenergic receptor and the regulatory elements necessary for expression of the DNA in the bacterial, yeast, or mammalian cell so located relative to the DNA encoding a human α_1 adrenergic receptor as to permit expression thereof. Suitable plasmids may include, but are not limited to plasmids adapted for expression in a mammalian cell, e.g., pCEXV-3 derived expression vector. Examples of such plasmids adapted for expression in a mammalian cell are plasmids comprising cDNA having coding sequences substantially the same as the coding sequence shown in Figures 1A-1I, 2A-2H, and 3A-3G and the regulatory elements necessary for expression of the DNA in the mammalian cell. These plasmids have been designated pcEXV- α_{1a} deposited under ATCC Accession No. 75319, pcEXV- α_{1b} deposited under ATCC Accession No. 75318, and pcEXV- α_{1c} deposited under ATCC

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Accession No. 75317, respectively . Those skilled in the art will readily appreciate that numerous plasmids adapted for expression in a mammalian cell which comprise DNA encoding human α_1 adrenergic receptors and the regulatory elements necessary to express such DNA in the mammalian cell may be constructed utilizing existing plasmids and adapted as appropriate to contain the regulatory elements necessary to express the DNA in the mammalian cell. The plasmids may be constructed by the methods described above for expression vectors and vectors in general, and by other methods well known in the art.

The deposits discussed supra were made pursuant to, and in satisfaction of, the provisions of the Budapest Treaty on the International Recognition of the Deposit of Microorganisms for the Purpose of Patent Procedure with the American Type Culture Collection (ATCC), 12301 Parklawn Drive, Rockville, Maryland 20852.

This invention provides a mammalian cell comprising a DNA molecule encoding a human α_1 adrenergic receptor, such as a mammalian cell comprising a plasmid adapted for expression in a mammalian cell, which comprises a DNA molecule encoding a human α_1 adrenergic receptor and the regulatory elements necessary for expression of the DNA in the mammalian cell so located relative to the DNA encoding a human α_1 adrenergic receptor as to permit expression thereof. Numerous mammalian cells may be used as hosts, including, but not limited to, the mouse fibroblast cell NIH3T3, CHO cells, HeLa cells, Ltk⁻ cells, human embryonic kidney cells, Cos cells, etc. Expression plasmids such as that described supra may be used to transfect mammalian cells by methods well known in the art such as calcium phosphate precipitation, or DNA encoding these human α_1 adrenergic receptors may be otherwise introduced into

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mammalian cells, e.g., by microinjection, to obtain mammalian cells which comprise DNA, e.g., cDNA or a plasmid, encoding a human α_1 adrenergic receptor.

5 This invention provides a nucleic acid probe comprising a nucleic acid molecule of at least 15 nucleotides capable of specifically hybridizing with a sequence included within the sequence of a nucleic acid molecule encoding a human α_{1a} adrenergic receptor, for example
10 with a coding sequence included within the sequence shown in Figures 1A-1I. This invention also provides a nucleic acid probe comprising a nucleic acid molecule of at least 15 nucleotides capable of specifically hybridizing with a sequence included within the
15 sequence of a nucleic acid molecule encoding a human α_{1b} adrenergic receptor, for example with a coding sequence included within the sequence shown in Figures 2A-2H. This invention also provides a nucleic acid probe comprising a nucleic acid molecule of at least 15
20 nucleotides capable of specifically hybridizing with a sequence included within the sequence of a nucleic acid molecule encoding a human α_{1c} adrenergic receptor, for example with a coding sequence included within the sequence shown in Figures 3A-3G. As used herein, the
25 phrase "specifically hybridizing" means the ability of a nucleic acid molecule to recognize a nucleic acid sequence complementary to its own and to form double-helical segments through hydrogen bonding between complementary base pairs. Nucleic acid probe
30 technology is well known to those skilled in the art who will readily appreciate that such probes may vary greatly in length and may be labeled with a detectable label, such as a radioisotope or fluorescent dye, to facilitate detection of the probe. Detection of
35 nucleic acid encoding a human α_1 adrenergic receptor is useful as a diagnostic test for any disease process in which levels of expression of the corresponding human

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α_{1a} , α_{1b} or α_{1c} adrenergic receptor are altered. DNA probe molecules are produced by insertion of a DNA molecule which encodes a human α_{1a} , human α_{1b} , or human α_{1c} adrenergic receptor or fragments thereof into

5 suitable vectors, such as plasmids or bacteriophages, followed by insertion into suitable bacterial host cells and replication and harvesting of the DNA probes, all using methods well known in the art. For example, the DNA may be extracted from a cell lysate using

10 phenol and ethanol, digested with restriction enzymes corresponding to the insertion sites of the DNA into the vector (discussed above), electrophoresed, and cut out of the resulting gel. Examples of such DNA molecules are shown in Figures 1A-1I, 2A-2H, and 3A-3G.

15 The probes are useful for "in situ" hybridization or in order to identify tissues which express this gene family, or for other hybridization assays for the presence of these genes or their mRNA in various biological tissues. In addition, synthesized

20 oligonucleotides (produced by a DNA synthesizer) complementary to the sequence of a DNA molecule which encodes a human α_{1a} adrenergic receptor, or complementary to the sequence of a DNA molecule which encodes a human α_{1b} adrenergic receptor or complementary

25 to the sequence of a DNA molecule which encodes a human α_{1c} adrenergic receptor are useful as probes for these genes, for their associated mRNA, or for the isolation of related genes by homology screening of genomic or cDNA libraries, or by the use of amplification

30 techniques such as the Polymerase Chain Reaction.

This invention also provides a method for detecting expression of a human α_{1a} adrenergic receptor on the surface of a cell by detecting the presence of mRNA

35 coding for a human α_{1a} adrenergic receptor. This invention also provides a method for detecting expression of a human α_{1b} adrenergic receptor on the

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surface of a cell by detecting the presence of mRNA coding for a human α_{1b} adrenergic receptor. This invention also provides a method for detecting expression of a human α_{1c} adrenergic receptor on the surface of a cell by detecting the presence of mRNA coding for a human α_{1c} adrenergic receptor. These methods comprise obtaining total mRNA from the cell using methods well known in the art and contacting the mRNA so obtained with a nucleic acid probe as described hereinabove, under hybridizing conditions, detecting the presence of mRNA hybridized to the probe, and thereby detecting the expression of a specific human α_1 adrenergic receptor by the cell. Hybridization of probes to target nucleic acid molecules such as mRNA molecules employs techniques well known in the art. However, in one embodiment of this invention, nucleic acids are extracted by precipitation from lysed cells and the mRNA is isolated from the extract using a column which binds the poly-A tails of the mRNA molecules (Maniatis, T. et al., Molecular Cloning; Cold Spring Harbor Laboratory, pp.197-98 (1982)). The mRNA is then exposed to radioactively labelled probe on a nitrocellulose membrane, and the probe hybridizes to and thereby labels complementary mRNA sequences. Binding may be detected by autoradiography or scintillation counting. However, other methods for performing these steps are well known to those skilled in the art, and the discussion above is merely an example.

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This invention provides an antisense oligonucleotide having a sequence capable of specifically binding with any sequences of an mRNA molecule which encodes a human α_{1a} adrenergic receptor so as to prevent translation of the human α_{1a} adrenergic receptor. This invention also provides an antisense oligonucleotide having a sequence capable of specifically binding with any sequences of

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an mRNA molecule which encodes a human α_{1b} adrenergic receptor so as to prevent translation of the human α_{1b} adrenergic receptor. This invention also provides an antisense oligonucleotide having a sequence capable of specifically binding with any sequences of an mRNA molecule which encodes a human α_{1c} adrenergic receptor so as to prevent translation of the human α_{1c} adrenergic receptor. As used herein, the phrase "specifically binding" means the ability of an antisense oligonucleotide to recognize a nucleic acid sequence complementary to its own and to form double-helical segments through hydrogen bonding between complementary base pairs. The antisense oligonucleotide may have a sequence capable of specifically binding with any sequences of the cDNA molecules whose sequences are shown in Figures 1A-1I, 2A-2H or 3A-3G. A particular example of an antisense oligonucleotide is an antisense oligonucleotide comprising chemical analogues of nucleotides which are known to one of skill in the art.

This invention also provides a pharmaceutical composition comprising an effective amount of the oligonucleotide described above effective to reduce expression of a human α_{1a} adrenergic receptor, by passing through a cell membrane and specifically binding with mRNA encoding the human α_{1a} adrenergic receptor in the cell so as to prevent its translation and a pharmaceutically acceptable hydrophobic carrier capable of passing through a cell membrane. This invention also provides a pharmaceutical composition comprising an effective amount of the oligonucleotide described above effective to reduce expression of a human α_{1b} adrenergic receptor in the cell so as to prevent its translation and a pharmaceutically acceptable hydrophobic carrier capable of passing through a cell membrane. This invention further provides a pharmaceutical composition comprising an

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effective amount of the oligonucleotide described above effective to reduce expression of a human α_{1c} adrenergic receptor in the cell so as to prevent its translation and a pharmaceutically acceptable hydrophobic carrier capable of passing through a cell membrane. As used herein, the term "pharmaceutically acceptable carrier" encompasses any of the standard pharmaceutical carriers, such as a phosphate buffered saline solution, water, and emulsions, such as an oil/water or water/oil emulsion, and various types of wetting agents. The oligonucleotide may be coupled to a substance which inactivates mRNA, such as a ribozyme. The pharmaceutically acceptable hydrophobic carrier capable of passing through cell membranes may also comprise a structure which binds to a transporter specific for a selected cell type and is thereby taken up by cells of the selected cell type. The structure may be part of a protein known to bind a cell-type specific transporter, for example an insulin molecule, which would target pancreatic cells. DNA molecules having coding sequences substantially the same as the coding sequence shown in Figures 1A-1I, 2A-2H, or 3A-3G may be used as the oligonucleotides of the pharmaceutical composition.

This invention also provides a method of treating abnormalities which are alleviated by reduction of expression of a human α_1 adrenergic receptor. This method comprises administering to a subject an effective amount of the pharmaceutical composition described above effective to reduce expression of the human α_1 adrenergic receptor by the subject. This invention further provides a method of treating an abnormal condition related to α_1 adrenergic receptor activity which comprises administering to a subject an amount of the pharmaceutical composition described above effective to reduce expression of the human α_1

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adrenergic receptor by the subject. Examples of such an abnormal condition include but are not limited to benign prostatic hypertrophy, coronary heart disease, hypertension, urinary retention, insulin resistance, atherosclerosis, sympathetic dystrophy syndrome, glaucoma, cardiac arrhythmias erectile dysfunction, and Renaud's syndrome.

Antisense oligonucleotide drugs inhibit translation of mRNA encoding the human α_1a , human α_1b or human α_1c adrenergic receptors. Synthetic antisense oligonucleotides, or other antisense chemical structures are designed to bind to mRNA encoding the human α_1a adrenergic receptor, to mRNA encoding the human α_1b adrenergic receptor or to mRNA encoding the human α_1c adrenergic receptor and inhibit translation of mRNA and are useful as drugs to inhibit expression of the human α_{1a} adrenergic receptor, the human α_{1b} adrenergic receptor or the human α_{1c} adrenergic receptor in patients. This invention provides a means to therapeutically alter levels of expression of the human α_{1a} adrenergic receptor, the human α_{1b} adrenergic receptor or the human α_{1c} adrenergic receptor by the use of a synthetic antisense oligonucleotide drug (SAOD) which inhibits translation of mRNA encoding these α_1 adrenergic receptors. Synthetic antisense oligonucleotides, or other antisense chemical structures designed to recognize and selectively bind to mRNA, are constructed to be complementary to portions of the nucleotide sequences shown in Figures 1A-1I, 2A-2H, or 3A-3G of DNA, RNA or of chemically modified, artificial nucleic acids. The SAOD is designed to be stable in the blood stream for administration to patients by injection, or in laboratory cell culture conditions, for administration to cells removed from the patient. The SAOD is designed to be capable of passing through cell

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membranes in order to enter the cytoplasm of the cell by virtue of physical and chemical properties of the SAOD which render it capable of passing through cell membranes (e.g., by designing small, hydrophobic SAOD chemical structures) or by virtue of specific transport systems in the cell which recognize and transport the SAOD into the cell. In addition, the SAOD can be designed for administration only to certain selected cell populations by targeting the SAOD to be recognized by specific cellular uptake mechanisms which bind and take up the SAOD only within certain selected cell populations. For example, the SAOD may be designed to bind to a transporter found only in a certain cell type, as discussed above. The SAOD is also designed to recognize and selectively bind to the target mRNA sequence, which may correspond to a sequence contained within the sequences shown in Figures 1A-1I, 2A-2H, or 3A-3G by virtue of complementary base pairing to the mRNA. Finally, the SAOD is designed to inactivate the target mRNA sequence by any of three mechanisms: 1) by binding to the target mRNA and thus inducing degradation of the mRNA by intrinsic cellular mechanisms such as mRNA target by interfering with the binding of translation-regulating factors or of other chemical structures, such as ribozyme sequences or reactive chemical groups, which either degrade or chemically modify the target mRNA. Synthetic antisense oligonucleotide drugs have been shown to be capable of the properties described above when directed against mRNA targets (J.S. Cohen, Trends in Pharm. Sci 10, 435 (1989); H.M. Weintraub, Sci. AM. January (1990) p. 40). In addition, coupling of ribozymes to antisense oligonucleotides is a promising strategy for inactivating target mRNA (N. Sarver et al., Science 247, 1222 (1990)). An SAOD serves as an effective therapeutic agent if it is designed to be administered to a patient by injection, or if the patient's target

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cells are removed, treated with the SAOD in the laboratory, and replaced in the patient. In this manner, an SAOD serves as a therapy to reduce human α_1 adrenergic receptor expression in particular target cells of a patient, in any clinical condition which may benefit from reduced expression of a specific human α_1 adrenergic receptor.

This invention provides an antibody directed to a human α_{1a} adrenergic receptor. This antibody may comprise, for example, a monoclonal antibody directed to an epitope of a human α_{1a} adrenergic receptor present on the surface of a cell, the epitope having an amino acid sequence substantially the same as an amino acid sequence for a cell surface epitope of the human α_{1a} adrenergic receptor included in the amino acid sequence shown in Figures 1A-1I. This invention also provides an antibody directed to a human α_{1b} adrenergic receptor. This antibody may comprise, for example, a monoclonal antibody directed to an epitope of a human α_{1b} adrenergic receptor present on the surface of a cell, the epitope having an amino acid sequence substantially the same as an amino acid sequence for a cell surface epitope of the human α_{1b} adrenergic receptor included in the amino acid sequence shown in Figures 2A-2H. This invention also provides an antibody directed to a human α_{1c} adrenergic receptor. This antibody may comprise, for example, a monoclonal antibody directed to an epitope of a human α_{1c} adrenergic receptor present on the surface of a cell, the epitope having an amino acid sequence substantially the same as an amino acid sequence for a cell surface epitope of the human α_{1c} adrenergic receptor included in the amino acid sequence shown in Figures 3A-3G. Amino acid sequences may be analyzed by methods well known to those skilled in the art to determine whether they produce hydrophobic or hydrophilic regions in the proteins which they build.

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In the case of cell membrane proteins, hydrophobic regions are well known to form the part of the protein that is inserted into the lipid bilayer which forms the cell membrane, while hydrophilic regions are located on the cell surface, in an aqueous environment.

Therefore antibodies to the hydrophilic amino acid sequences shown in Figures 1A-1I will bind to a surface epitope of the human α_1a adrenergic receptor, antibodies to the hydrophilic amino acid sequences shown in Figures 2A-2H will bind to a surface epitope of a human α_1b adrenergic receptor, and antibodies to the hydrophilic amino acid sequences shown in Figures 3A-3G will bind to a surface epitope of a human α_1c adrenergic receptor as described. Antibodies directed to human α_1 adrenergic receptors may be serum-derived or monoclonal and are prepared using methods well known in the art. For example, monoclonal antibodies are prepared using hybridoma technology by fusing antibody producing B cells from immunized animals with myeloma cells and selecting the resulting hybridoma cell line producing the desired antibody. Cells such as NIH3T3 cells or Ltk⁺ cells may be used as immunogens to raise such an antibody. Alternatively, synthetic peptides may be prepared using commercially available machines and the amino acid sequence shown in Figures 1A-1I, 2A-2H, and 3A-3G. As a still further alternative DNA, such as a cDNA or a fragment thereof, may be cloned and expressed and the resulting polypeptide recovered and used as an immunogen. These antibodies are useful to detect the presence of human α_1 adrenergic receptors encoded by the isolated DNA, or to inhibit the function of α_1 adrenergic receptors in living animals, in humans, or in biological tissues or fluids isolated from animals or humans.

This invention provides a pharmaceutical composition which comprises an effective amount of an antibody

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directed to an epitope of a human α_{1a} adrenergic receptor and a pharmaceutically acceptable carrier. A monoclonal antibody directed to an epitope of a human α_{1a} adrenergic receptor present on the surface of a cell which has an amino acid sequence substantially the same as an amino acid sequence for a cell surface epitope of the human α_{1a} adrenergic receptor present on the surface of a cell which has an amino acid sequence substantially the same as an amino acid sequence for a cell surface epitope of the human α_{1a} adrenergic receptor included in the amino acid sequence shown in Figures 1A-1I is useful for this purpose. This invention also provides a pharmaceutical composition which comprises an effective amount of an antibody directed to an epitope of a human α_{1b} adrenergic receptor, effective to block binding of naturally occurring substrates to the human α_{1b} adrenergic receptor and a pharmaceutically acceptable carrier. A monoclonal antibody directed to an epitope of a human α_{1b} adrenergic receptor present on the surface of a cell which has an amino acid sequence substantially the same as an amino acid sequence for a cell surface epitope of the human α_{1b} adrenergic receptor included in the amino acid sequence shown in Figures 2A-2H is useful for this purpose. This invention provides a pharmaceutical composition which comprises an effective amount of an antibody directed to an epitope of a human α_{1c} adrenergic receptor effective to block binding of naturally occurring substrates to the human α_{1c} adrenergic receptor and a pharmaceutically acceptable carrier. A monoclonal antibody directed to an epitope of a human α_{1c} adrenergic receptor present on the surface of the cell which has an amino acid sequence substantially the same as an amino acid sequence for a cell surface epitope of the human α_{1c} adrenergic receptor included in the amino acid sequence shown in Figures 3A-3G is useful for this purpose.

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This invention also provides a method of treating abnormalities in a subject which are alleviated by reduction of expression of a specific human α_1 adrenergic receptor. The method comprises administering to the subject an effective amount of the pharmaceutical composition described above effective to block binding of naturally occurring substrates to the human α_1 adrenergic receptor and thereby alleviate abnormalities resulting from overexpression of the human α_1 adrenergic receptor. Binding of the antibody to the human α_1 adrenergic receptor from functioning, thereby neutralizing the effects of overexpression. The monoclonal antibodies described above are useful for this purpose. This invention additionally provides a method of treating an abnormal condition related to an excess of a specific human α_1 adrenergic receptor activity which comprises administering to a subject an amount of the pharmaceutical composition described above effective to block binding of naturally occurring substrates to the human α_1 adrenergic receptor and thereby alleviate the abnormal condition. Examples of such an abnormal condition include but are not limited to benign prostatic hypertrophy, coronary heart disease, insulin resistance, atherosclerosis, sympathetic dystrophy syndrome, glaucoma, cardiac arrhythmias, hypertension, urinary retention, erectile dysfunction, and Renaud's syndrome.

This invention provides methods of detecting the presence of a specific human α_1 adrenergic receptor on the surface of a cell which comprises contacting the cell with an antibody directed to a specific human α_1 adrenergic receptor, under conditions permitting binding of the antibody to the human α_1 adrenergic receptor, under conditions permitting binding of the antibody to the human α_1 adrenergic receptor, detecting the presence of any antibody bound to the α_1 adrenergic

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receptor, and thereby the presence of the specific human α_1 adrenergic receptor on the surface of the cell. Such methods are useful for determining whether a given cell is defective in expression of a specific human α_1 adrenergic receptor. Bound antibodies are detected by methods well known in the art, for example by binding fluorescent markers to the antibodies and examining the cell sample under a fluorescence microscope to detect fluorescence on a cell indicative of antibody binding. The monoclonal antibodies described above are useful for this purpose.

This invention provides a transgenic nonhuman mammal comprising DNA encoding a human α_{1a} adrenergic receptor. This invention also provides a transgenic nonhuman mammal comprising DNA encoding a human α_{1b} adrenergic receptor. This invention also provides a transgenic nonhuman mammal comprising DNA encoding a human α_{1c} adrenergic receptor.

This invention also provides a transgenic nonhuman mammal comprising DNA encoding a human α_{1a} adrenergic receptor so mutated as to be incapable of normal human α_{1a} adrenergic receptor activity, and not expressing native human α_{1a} adrenergic receptor activity, and not expressing native human α_{1a} adrenergic receptor. This invention also provides a transgenic nonhuman mammal comprising DNA encoding a human α_{1b} adrenergic receptor so mutated as to be incapable of normal human α_{1b} adrenergic receptor activity, and not expressing native human α_{1b} adrenergic receptor. This invention also provides a transgenic nonhuman mammal comprising DNA encoding a human α_{1c} adrenergic receptor so mutated as to be incapable of normal human α_{1c} adrenergic receptor activity, and not expressing native human α_{1c} adrenergic receptor.

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This invention provides a transgenic non-human animal whose genome comprises DNA encoding a human α_{1a} adrenergic receptor so placed as to be transcribed into antisense mRNA which is complementary to mRNA encoding a human α_{1a} adrenergic receptor thereby reducing its translation. This invention also provides a transgenic nonhuman mammal whose genome comprises DNA encoding a human α_{1b} adrenergic receptor so placed as to be transcribed into antisense mRNA which is complementary to mRNA encoding the human α_{1b} adrenergic receptor and which hybridizes to mRNA encoding a human α_{1b} adrenergic receptor thereby reducing its translation. This invention provides a transgenic non-human animal whose genome comprises DNA encoding a human α_{1c} adrenergic receptor so placed as to be transcribed into antisense mRNA which is complementary to mRNA encoding a human α_{1c} adrenergic receptor and which hybridizes to mRNA encoding the human α_{1c} adrenergic receptor thereby reducing its translation. The DNA may additionally comprise an inducible promoter or additionally comprise tissue specific regulatory elements, so that expression can be induced, or restricted to specific cell types. Examples of DNA are DNA or cDNA molecules having a coding sequence substantially the same as the coding sequences shown in Figures 1A-1I, 2A-2H, or 3A-3G. An example of a transgenic animal is a transgenic mouse. Examples of tissue specificity-determining regions are the metallothionein promoter (Low, M.J., Lechan, R.M., Hammer, R.E. et al. Science 231:1002-1004 (1986) and the L7 promoter (Oberdick, J., Smeyne, R.J., Mann, J.R., Jackson, S. and Morgan, J.I. Science 248:223-226 (1990)).

Animal model systems which elucidate the physiological and behavioral roles of human α_1 adrenergic receptors are produced by creating transgenic animals in which the increased or decreased, or the amino acid sequence

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of the expressed α_1 adrenergic receptor is altered, by a variety of techniques. Examples of these techniques include, but are not limited to: 1) Insertion of normal or mutant versions of DNA encoding a human α_1 adrenergic receptor or homologous animal versions of these genes, by microinjection, retroviral infection or other means well known to those skilled in the art, into appropriate fertilized embryos in order to produce a transgenic animal (Hogan B et al., Manipulating the Mouse Embryo, A Laboratory Manual, Cold Spring Harbor Laboratory (1986)) or, 2) Homologous recombination (Capecchi M.R. Science 244:1288-1292 (1989); Zimmer, A. and Gruss, P. Nature 338:150-153 (1989)) of mutant or normal, human or animal version of the genes encoding α_1 adrenergic receptors with the native gene locus in transgenic animals to alter the regulation of expression or the structure α_1 of these α_1 adrenergic receptors. The technique of homologous α_1 adrenergic receptors. The technique of homologous recombination is well known in the art. It replaces the native gene with the inserted gene and so is useful for producing an animal that cannot express native α_1 adrenergic receptor but does express, for example an inserted mutant human α_1 adrenergic receptor, which has replaced the native α_1 adrenergic receptor in the animal's genome by recombination, resulting in underexpression of the α_1 adrenergic receptor. Microinjection adds genes to the genome, but does not remove them, and so is useful for producing an animal which expresses its own and added α_1 adrenergic receptors, resulting in overexpression of the α_1 adrenergic receptor.

One means available for producing a transgenic animal, with a mouse as an example, is as follows: Female mice are mated, and the resulting fertilized eggs are dissected out of their oviducts. The eggs are stored in an appropriate medium such as M2 medium (Hogan B et

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al., Manipulating the Mouse Embryo, A Laboratory Manual, Cold Spring Harbor Laboratory (1986)). DNA or cDNA encoding a human α_1 adrenergic receptor is purified from a vector (such as plasmids pCEXV- α_{1b} , or pCEXV- α_{1c} described above) by methods well known in the art. Inducible promoters may be fused with the coding region of the DNA to provide an experimental means to regulate expression of the trans-gene. Alternatively or in addition, tissue specific regulatory elements may be fused with the coding region to permit tissue-specific expression of the trans-gene. The DNA, in an appropriately buffered solution, is put into a microinjection needle (which may be made from capillary tubing using a pipet puller) and the egg to be injected is put in a depression slide. The needle is inserted into the pronucleus of the egg, and the DNA solution is injected. The injected egg is then transferred into the oviduct of a pseudopregnant mouse (a mouse stimulated by the appropriate hormones to maintain pregnancy but which is not actually pregnant), where it proceeds to the uterus, implants, and develops to term. As noted above, microinjection is not the only method for inserting DNA into the egg cell, and is used here only for exemplary purposes.

Since the normal action of α_1 adrenergic-specific drugs is to activate or to inhibit the α_1 adrenergic receptor, the transgenic animal model systems described above are useful for testing the biological activity of drugs directed against specific human α_1 adrenergic receptors even before such drugs become available. These animal model systems are useful for predicting or evaluating possible therapeutic applications of drugs which activate or inhibit these human α_1 adrenergic receptors by inducing or inhibiting expression of the native or transgene and thus increasing or decreasing expression of normal or mutant human α_1 adrenergic

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receptor in the living animal. Thus, a model system is produced in which the biological activity of drugs directed against these human α_1 adrenergic receptors are evaluated before such drugs become available. The

5 transgenic animals which over or under produce a specific human α_1 adrenergic over or under produce a specific human α_1 adrenergic over or under produce a specific human α_1 adrenergic receptor indicate by their physiological state whether over or under production of

10 the human α_1 adrenergic receptor is therapeutically useful. It is therefore useful to evaluate drug action based on the transgenic model system. One use is based on the fact that it is well known in the art that a drug such as an antidepressant acts by blocking

15 neurotransmitter uptake, and thereby increases the amount of neurotransmitter in the synaptic cleft. The physiological result of this action is to stimulate the production of less human α_1 adrenergic receptor by the affected cells, leading eventually to underexpression.

20 Therefore, an animal which underexpresses human α_1 adrenergic receptor is useful as a test system to investigate whether the actions of such drugs which result in under expression are in fact therapeutic. Another use is that if overexpression is found to lead

25 abnormalities, then a drug which down-regulates or acts as an antagonist to the human α_1 adrenergic receptor is indicated as worth developing, and if a promising therapeutic application is uncovered by these animal model systems, activation or inhibition of the specific

30 human α_1 adrenergic receptor or antagonist drugs directed against these human α_1 adrenergic receptors or by any method which increases or decreases the expression of these α_1 adrenergic receptors in man.

35 Further provided by this invention is a method of determining the physiological effects of expressing varying levels of a human α_1 adrenergic receptor which

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comprises producing a transgenic nonhuman animal whose levels of α_1 adrenergic receptor expression are varied by use of an inducible promoter which regulates human α_1 adrenergic receptor expression. This invention also provides a method for determining the physiological effects of expressing varying levels of human α_1 adrenergic receptors which comprise producing a panel of transgenic nonhuman animals each expressing a different amount of a human α_1 adrenergic receptor. Such animals may be produced by introducing different amounts of DNA encoding a human α_1 adrenergic receptor into the oocytes from which the transgenic animals are developed.

This invention also provides a method for identifying a substance capable of alleviating abnormalities resulting from overexpression of a human α_1 adrenergic receptor comprising administering the substance to a transgenic nonhuman mammal expressing at least one artificially introduced DNA molecule encoding a human α_1 adrenergic receptor and determining whether the substance alleviates the physical and behavioral abnormalities displayed by the transgenic nonhuman mammal as a result of overexpression of a human α_1 adrenergic receptor. As used herein, the term "substance" means a compound or composition which may be natural, synthetic, or a product derived from screening. Examples of DNA molecules are DNA or cDNA molecules having a coding sequence substantially the same as the coding sequences shown in Figures 1A-1I, 2A-2H, or 3A-3G.

This invention provides a pharmaceutical composition comprising an amount of the substance described supra effective to alleviate the abnormalities resulting from overexpression of a human α_1 adrenergic receptor and a pharmaceutically acceptable carrier. This invention

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provides a pharmaceutical composition comprising an amount of the substance described supra effective to alleviate the abnormalities resulting from overexpression of a human α_{1b} adrenergic receptor and a
5 pharmaceutically acceptable carrier. This invention also provides a pharmaceutical composition comprising an amount of the substance described supra effective to alleviate the abnormalities resulting from overexpression of a human α_{1c} adrenergic receptor and a
10 pharmaceutically acceptable carrier.

This invention further provides a method for treating the abnormalities resulting from overexpression of a human α_1 adrenergic receptor which comprises
15 administering to a subject an amount of the pharmaceutical composition described above effective to alleviate the abnormalities resulting from overexpression of the human α_1 adrenergic receptor.

20 This invention provides a method for identifying a substance capable of alleviating the abnormalities resulting from underexpression of a human α_1 adrenergic receptor comprising administering the substance to the transgenic nonhuman mammal described above which
25 expresses only a nonfunctional human α_1 adrenergic receptor and determining whether the substance alleviates the physical and behavioral abnormalities displayed by the transgenic nonhuman mammal as a result of underexpression of the human α_1 adrenergic receptor.

30 This invention also provides a pharmaceutical composition comprising an amount of a substance effective to alleviate abnormalities resulting from underexpression of a human α_1 adrenergic receptor and
35 a pharmaceutically acceptable carrier.

This invention also provides a method for treating the

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abnormalities resulting from underexpression of a human α_1 adrenergic receptor which comprises administering to a subject an amount of the pharmaceutical composition described above effective to alleviate the abnormalities resulting from underexpression of a human α_1 adrenergic receptor.

This invention provides a method for diagnosing a predisposition to a disorder associated with the expression of a specific human α_1 adrenergic receptor allele which comprises: a) obtaining DNA of subjects suffering from the disorder; b) performing a restriction digest of the DNA with a panel of restriction enzymes; c) electrophoretically separating the resulting DNA fragments on a sizing gel; d) contacting the resulting gel with a nucleic acid probe capable of specifically hybridizing to DNA encoding a human α_1 adrenergic receptor and labelled bands which have hybridized to the DNA encoding a human α_1 adrenergic receptor labelled with a detectable marker to create a unique band pattern specific to the DNA of subjects suffering from the disorder; f) preparing DNA obtained for diagnosis by steps a-e; and g) comparing the unique band pattern specific to the DNA of subjects suffering from the disorder from step e and the DNA obtained for diagnosis from step f to determine whether the patterns are the same or different and thereby to diagnose predisposition to the disorder if the patterns are the same. This method may also be used to diagnose a disorder associated with the expression of a specific human α_1 adrenergic receptor allele.

This invention provides a method of preparing an isolated human α_1 adrenergic receptor which comprises inducing cells to express the human α_1 adrenergic receptor, recovering the α_1 adrenergic receptor from the resulting cells, and purifying the α_1 adrenergic

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receptor so recovered. An example of an isolated human α_{1a} adrenergic receptor is an isolated protein having substantially the same amino acid sequence as the amino acid sequence shown in Figures 1A-1I. An example of an isolated human α_{1b} adrenergic receptor is an isolated protein having substantially the same amino acid sequence as the amino acid sequence shown in Figures 1A-1I. An example of an isolated human α_{1c} adrenergic receptor is an isolated protein having substantially the same amino acid sequence shown in Figure 2A-2H. An example of an isolated human α_{1c} adrenergic receptor is an isolated protein having substantially the same amino acid sequence shown in Figure 3A-3G. For example, cells can be induced to express human α_1 adrenergic receptor by exposure to substances such as hormones. The cells can then be homogenized and the human α_1 adrenergic receptor isolated from the homogenate using an affinity column comprising, for example, epinephrine, norepinephrine, or another substance which is known to bind to the human α_1 adrenergic receptor. The resulting fractions can then be purified by contacting them with an ion exchange column, and determining which fraction contains human α_1 adrenergic receptor activity or binds anti-human α_1 adrenergic receptor activity or binds anti-human α_1 adrenergic receptor antibodies.

This invention provides a method of preparing the isolated human α_{1a} adrenergic receptor which comprises inserting nucleic acid encoding the human α_{1a} adrenergic receptor in a suitable vector, inserting the resulting vector in a suitable host cell, recovering the α_{1a} adrenergic receptor produced by the resulting cell, and purifying the α_{1a} adrenergic receptor so recovered. An example of an isolated human α_{1a} adrenergic receptor is an isolated protein having substantially the same amino acid sequence as the amino acid sequence shown in

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Figures 1A-1I. This invention also provides a method of preparing the isolated human α_{1b} adrenergic receptor which comprises inserting nucleic acid encoding the human α_{1b} adrenergic receptor in a suitable vector, inserting the resulting vector in a suitable host, recovering the α_{1b} adrenergic receptor produced by the resulting cell, and purifying the α_{1c} adrenergic receptor so recovered. These methods for preparing human α_1 adrenergic receptor uses recombinant DNA technology methods well known in the art. For example, isolated nucleic acid encoding a human α_1 adrenergic receptor is inserted in a suitable vector, such as an expression vector. A suitable host cell, such as a bacterial cell, or a eukaryotic cell such as a yeast cell is transfected with the vector. The human α_1 adrenergic receptor is isolated from the culture medium by affinity purification or by chromatography or by other methods well known in the art.

This invention provides a method of determining whether a ligand not known to be capable of binding to a human α_1 adrenergic receptor can bind to a human α_1 adrenergic receptor, which comprises contacting a mammalian cell comprising a plasmid adapted for expression in a mammalian cell which further comprises a DNA molecule which expresses a human α_1 adrenergic receptor on the cell surface with the ligand under conditions permitting binding of ligands known to bind to the human α_1 adrenergic receptor, detecting the presence of any ligand bound to the human α_1 adrenergic receptor. The DNA in the cell may have a coding sequence substantially the same as the coding sequences shown in Figures 1A-1I, 2A-2h, or 3A-3G, preferably, the mammalian cell is nonneuronal in origin. An example of a nonneuronal mammalian cell is a Cos7 cell. The preferred method for determining whether a ligand is capable of binding to the human α_1 adrenergic

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receptor comprises contacting a transfected nonneuronal mammalian cell (i.e. a cell that does not naturally express any type of human α_1 adrenergic receptor, thus will only express such human α_1 adrenergic receptor if it is transfected into the cell) expressing a human α_1 adrenergic receptor on its surface, or contacting a membrane preparation derived from such a transfected cell, with the ligand under conditions which are known to prevail, and thus be associated with in vivo binding of the substrates to a human α_1 adrenergic receptor, detecting the presence of any of the ligand being tested bound to the human α_1 adrenergic receptor on the surface of the cell, and thereby determining whether the ligand binds to the human α_1 adrenergic receptor. This response system is obtained by transfection of isolated DNA into a suitable host cell. Such a host system might be isolated from pre-existing cell lines, or can be generated by inserting appropriate components into existing cell lines. Such a transfection system provides a complete response system for investigation or assay of the functional activity of human α_1 adrenergic receptors with ligands as described above. Transfection systems are useful as living cell cultures for competitive binding assays between known or candidate drugs and substrates which bind to the human α_1 adrenergic receptor and which are labeled by radioactive, spectroscopic or other reagents. Membrane preparations containing the transporter isolated from transfected cells are also useful for these competitive binding assays. A transfection system constitutes a "drug discovery system" useful for the identification of natural or synthetic compounds with potential for drug development that can be further modified or used directly as therapeutic compounds to activate or inhibit the natural functions of a specific human α_1 adrenergic receptor. The transfection system is also useful for determining the affinity and efficacy of

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known drugs at human α_1 adrenergic receptor binding sites.

5 This invention provides a method for identifying a
ligand which interacts with, and activates or blocks
the activation of, a human α_1 adrenergic receptor on
the surface of the cell, which comprises contacting a
mammalian cell which comprises a plasmid adapted for
10 expression in a mammalian cell which further comprises
a DNA molecule which expresses a human α_1 adrenergic
receptor on the cell surface with the ligand,
determining whether the ligand activates or blocks the
activation of the receptor using a bioassay such as a
15 second messenger assays, and thereby identifying a
ligand which interacts with, and activates or blocks
the activation of, a human α_1 adrenergic receptor.

20 This invention provides functional assays for
identifying ligands and drugs which bind to and
activate or inhibit a specific human α_1 adrenergic
receptor activity.

25 This invention provides a method for identifying a
ligand which is capable of binding to and activating or
inhibiting a human α_1 adrenergic receptor, which
comprises contacting a mammalian cell, wherein the
membrane lipids have been labelled by prior incubation
with a labelled myo-inositol phosphate molecule, the
mammalian cell comprising a plasmid adapted for
30 expression in a mammalian cell which further comprises
a DNA molecule which expresses a human α_1 adrenergic
receptor with the ligand and identifying an inositol
phosphate metabolite released from the membrane lipid
as a result of ligand binding to and activating an α_1
35 adrenergic receptor.

This invention provides method for identifying a ligand

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that is capable of binding to and activating or inhibiting a human α_1 adrenergic receptor, where in the binding of ligand to the adrenergic receptor results in a physiological response, which comprises contacting a mammalian cell which further comprises a DNA molecule which expresses a human α_1 adrenergic receptor with a calcium sensitive fluorescent indicator, removing the indicator that has not been taken up by the cell, contacting the cells with the ligand and identifying an increase or decrease in intracellular Ca^{+2} as a result of ligand binding to and activating receptors.

Transformed mammalian cells for identifying the ligands and drugs that affect the functional properties of the human α adrenergic receptor include 292- α_1 -10, C- α_1 b-6 and C- α_1 c-7.

This invention also provides a method of screening drugs to identify drugs which interact with, and bind to, a human α_1 adrenergic receptor on the surface of a cell, which comprises contacting a mammalian cell which comprises a plasmid adapted for expression in a mammalian cell which further comprises a DNA molecule which expresses a human α_1 adrenergic receptor on the cell surface with a plurality of drugs, determining those drugs which bind to the human α_1 adrenergic receptor expressed on the cell surface of the mammalian cell, and thereby identifying drugs which interact with, and bind to, the human α_1 adrenergic receptor. Various methods of detection may be employed. The drugs may be "labeled" by association with a detectable marker substance (e.g., radiolabel or a non-isotopic label such as biotin). The DNA in the cell may have a coding sequence substantially the same as the coding sequences shown in Figures 1A-1I, 2A-2H or 3A-3G. Preferably, the mammalian cell is nonneuronal in origin. An example of a nonneuronal mammalian cell is

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a Cos7 cell. Drug candidates are identified by choosing chemical compounds which bind with high affinity to the human α_1 adrenergic receptor expressed on the cell surface in transfected cells, using
5 radioligand binding methods well known in the art, examples of which are shown in the binding assays described herein. Drug candidates are also screened for selectivity by identifying compounds which bind with high affinity to one particular human α_1
10 adrenergic receptor subtype but do not bind with high affinity to any other human α_1 adrenergic receptor subtype or to any other known receptor site. Because selective, high affinity compounds interact primarily with the target human α_1 adrenergic site after
15 administration to the patient, the chances of producing a drug with unwanted side effects are minimized by this approach. This invention provides a pharmaceutical composition comprising a drug identified by the method described above and a pharmaceutically acceptable
20 carrier. As used herein, the term "pharmaceutically acceptable carrier" encompasses any of the standard pharmaceutical carriers, such as a phosphate buffered saline solution, water, and emulsions, such as an oil/water or water/oil emulsion, and various types of
25 wetting agents. Once the candidate drug has been shown to be adequately bio-available following a particular route of administration, for example orally or by injection (adequate therapeutic concentrations must be maintained at the site of action for an adequate period
30 to gain the desired therapeutic benefit), and has been shown to be non-toxic and therapeutically effective in appropriate disease models, the drug may be administered to patients by that route of administration determined to make the drug bio-
35 available, in an appropriate solid or solution formulation, to gain the desired therapeutic benefit.

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This invention also provides a method for treating an abnormal condition related to an excess of activity of a human α_1 adrenergic receptor subtype, which comprises administering a patient an amount of a pharmaceutical composition described above, effective to reduce α_1 adrenergic activity as a result of naturally occurring substrate binding to and activating a specific α_1 adrenergic receptor. Examples of such abnormalities related to an excess of activity of a human α_1 adrenergic receptor subtype include but are limited to benign prostatic hypertrophy, coronary heart disease, hypertension, urinary retention, insulin resistance, atherosclerosis, sympathetic dystrophy syndrome, glaucoma, cardiac arrhythmias erectile dysfunction, and Renaud's syndrome.

This invention also provides a method of treating abnormalities which are alleviated by an increase in the activity of a specific human α_1 adrenergic receptor, which comprises administering a patient an amount of a pharmaceutical composition described above, effective to increase the activity of the specific human α_1 adrenergic receptor thereby alleviating abnormalities resulting from abnormally low receptor activity. Examples of such abnormalities related to a decrease in the activity of a specific human α_1 adrenergic receptor include but are not limited to congestive heart failure, urinary incontinence, nasal congestion and hypotension.

Applicants have identified individual human α_1 adrenergic receptor subtypes and have described methods for the identification of pharmacological compounds for therapeutic treatments. Pharmacological compounds which are directed against a specific human adrenergic receptor subtype provide effective new therapies with minimal side effects.

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Elucidation of the molecular structures of the neuronal human α_1 adrenergic receptors transporters is an important step in the understanding of α -adrenergic neurotransmission. This disclosure reports the isolation, the nucleic acid sequence, and functional expression of DNA clones isolated from human brain which encode human α_1 adrenergic receptor. The identification of these human α_1 adrenergic receptor will play a pivotal role in elucidating the molecular mechanisms underlying α -adrenergic transmission, and should also aid in the development of novel therapeutic agents.

DNA clones encoding human α_1 adrenergic receptor have been isolated from human brain, and their functional properties have been examined in mammalian cells.

This invention identifies for the first time three new human α_1 adrenergic receptor, their amino acid sequences, and their human genes. The information and experimental tools provided by this discovery are useful to generate new therapeutic agents, and new therapeutic or diagnostic assays for these new human receptors, their associated mRNA molecules or their associated genomic DNAs. The information and experimental tools provided by this discovery will be useful to generate new therapeutic agents, and new therapeutic or diagnostic assays for these new human receptors, their associates mRNA molecules, or their associated genomic DNAs.

Specifically, this invention relates to the first isolation of human DNA clones encoding three α_1 -adrenergic receptor. In addition, the human α_1 adrenergic receptor have been expressed in mammalian cells by transfecting the cells with the plasmids pCEXV- α_{1a} , pCEXV- α_{1c} . The pharmacological binding

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properties of these receptor proteins have been determined, and these binding properties classify these receptor proteins as α_1 adrenergic receptor. Mammalian cell lines expressing the human α_1 adrenergic receptor on the cell surface have been constructed, thus establishing the first well-defined, cultured cell lines with which to study human α_1 adrenergic receptor. Examples of transformed mammalian cells, expressing human α_1 adrenergic receptor are L- α -1a, expressing a human α_{1a} adrenergic receptor, L- α 1b expressing a human α_{1b} adrenergic receptor, and L- α 1c expressing a human α_{1c} adrenergic receptor. These cells are suitable for studying the pharmacological properties of the human α_1 adrenergic receptor and for the screening of ligands and drugs that specifically bind to human α_1 adrenergic receptor subtypes.

The invention will be better understood by reference to the Experimental Details which follow, but those skilled in the art will readily appreciate that the specific experiments detailed are only illustrative, and are not meant to limit the invention as described herein, which is defined by the claims which follow thereafter.

25

MATERIALS AND METHODS

Cloning and Sequencing

5 α1a: A human lymphocyte genomic library in λ dash II
 (≈1.5 x 10⁶ total recombinants; Stratagene, LaJolla,
 CA.) was screened using a cloned rat PCR fragment
 (RBNC2) as a probe. RBNC2 was obtained by amplifying
10 randomly primed rat brain cDNA with degenerate primers
 designed to conserved regions of transmembrane (Tm)
 regions 2 and 6 of serotonin receptors. The sequence
 of one PCR product, RBNC2, exhibited strong homology to
 the α1 AR family.

15 The probe was labeled with [³² P] by the method of
 random priming (5) (Prime-It Random Primer kit,
 Stratagene, LaJolla, CA.). Hybridization was performed
 at 40°C. in a solution containing 50% formamide, 10%
 dextran sulfate, 5x SSC (1X SSC is 0.15M sodium
20 chloride, 0.015M sodium citrate), 1x Denhardt's
 solution (0.02% polyvinylpyrrolidone, 0.02% Ficoll,
 0.02% bovine serum albumin), and 200 μg/μl sonicated
 salmon sperm DNA. The filters were washed at 50°C. in
 0.1x SSC containing 0.5% sodium dodecyl sulfate and
25 exposed at -70°C to Kodak XAR film in the presence of
 an intensifying screen. Lambda phage clones
 hybridizing with the probe were plaque purified and DNA
 was prepared for Southern blot analysis (22, 17). For
 subcloning and further Southern blot analysis, DNA was
30 cloned into pUC18 (Pharmacia, Piscataway, NJ) or
 pBluescript (Stratagene, LaJolla, Ca.). Nucleotide
 sequence analysis was accomplished by the Sanger
 dideoxy nucleotide chain termination method (18) on
 denatured double-stranded plasmid templates, using
 Sequenase (US Biochemical Corp., Cleveland, OH), Bst
35 DNA sequencing kit (Bio-Rad Laboratories, Richmond,
 CA.), or TaqTrack sequencing kit (Promega Corporation,
 Madison, WI.).

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In order to isolate a full-length clone, human cDNA libraries were screened by polymerase chain reaction (PCR) with 1 μ M each of specific oligonucleotide primers designed off the isolated genomic clone: from the sense strand (nucleotide 598-626), 5' CACTCAAGTACCCAGCCATCATGAC 3' and from the antisense strand (nucleotide 979-1003), 5' CGGAGAGCGAGCTGCGGAAGGTGTG 3' (see Figures 1A01I). The primers were from non-conserved portions of the receptor gene, specifically in the Tm3-Tm3 loop and in the Tm5-Tm6 loop regions for the upstream and downstream primers, respectively. One to 2 μ l of phage DNA from cDNA libraries (λ ZapII; Stratagene, LaJolla, CA.), representing $\approx 10^6$ - 10^7 pfu, were amplified in 10mM Tris-HCl, pH 8.3, 50mM KCl, 1.5mM MgCl₂, 0.01% gelatin, 200 μ M each dATP, dCTP, dTTP, 2.5 units of *Thermus aquaticus* DNA polymerase (Taq polymerase; Perkin-Elmer-Cetus, Norwalk, CT.). The amplification profile was run for 30 cycles: a 5 min. initial (i.e. 1 cycle denaturation at 95°C., followed by 2 min. at 94°C., 2 min at 68°C., and 3 min at 72°C., with a 3 sec. extension, followed by a final 10 min. extension at 72°C. PCR products were analyzed by ethidium bromide (EtBr) stained agarose gels and any sample exhibiting a band on the EtBr stained gel was considered positive.

A positive library was then plated and screened with overlapping 45-mer oligonucleotide probes, filled-in using [α -³²P]dCTP and [α -³²P]dATP and Klenow fragment of DNA polymerase. This probe was internal to the amplification primers discussed above from the sense strand (nucleotide 890 - 934), 5' GCAAGGCCTCCGAGGTGGTGCTGCGCATCCACTGTGCGGCGCGG 3', and from the anti-sense strand (nucleotide 915-961), 5' TGCCGTGCGCCCCGTGCGGCGCCGTGGCCGCGCCGCGACAGTGGATG 3' (see Figures 1A-1I). Positive cDNA phage clones were plaque certified and pBluescript recombinant DNAs were

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excision-rescued from λ Zap II using helper phage R408, as described by manufacturer's protocol (Stratagene, LaJolla, CA.). Insert size was confirmed by restriction enzyme digest analysis and recombinants were sequences as described above.

$\alpha 1b$: A human placenta genomic library in λ dash II ($\approx 1.5 \times 10^6$ total recombinants; Stratagene, LaJolla, CA.) was screened using overlapping 45-mer oligonucleotides radiolabeled as described above and directed to the third, fifth and sixth transmembrane regions of serotonin 5HT1D β receptor gene. Hybridization and washing conditions were identical to that described for $\alpha 1a$ above except lower stringency hybridization and washes were conducted; specifically, hybridization in 25% formamide and washes at 40°C.

Positive-hybridizing λ phage clones were plaque-purified, analyzed by Southern blot analysis, subcloned and sequenced, as described above for $\alpha 1a$. In order to isolate full-length clones, human cDNA libraries in λ Zap II (Stratagene, LaJolla, CA.) were screened by polymerase chain reaction as described above. The upstream and downstream PCR primers used were from the Tm40Tm5 loop and the Tm5-Tm6 loop, respectively: from the sense strand (nucleotide 567-593), 5' CAACGATGACAAGGA GTGCGGGGTCAC 3', and from the antisense strand (nucleotide 822-847), 5' TTTGACAGCTATGGAACCTCCTGGGG 3' (see Fig. 2). PCR, library screen, plaque purification excision-rescue from λ Zap II, restriction digestions and sequencing were accomplished as described above for $\alpha 1a$. The internal probe was: from the sense strand (nucleotide 745-789), 5' AAGGAGCTGACCCTGAGGATCCATTCCAAGAACTTTC ACGAGGAC 3', and from the anti-sense strand (nucleotide 770-814), 5' CCTTGGCCTTGGTACTGCTAAGGGTGTCTCGTGAA GTTCTTGG 3' (see Figures 2A-2H).

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α lc: A human lymphocyte genomic library in λ dash II ($\approx 1.5 \times 10^6$ total recombinants; Stratagene, LaJolla, CA.) was screened using overlapping 45-mer oligonucleotides radiolabeled as described for α la and directed to the third, fifth and sixth transmembrane regions of serotonin 5HT1A receptor gene. Hybridization and washing conditions were identical to that described for α lb. Positive-hybridizing λ phage clones were plaque-purified, analyzed by Southern blot analysis, subcloned and sequenced, as described above for α la. Identification and isolation of full-length clones by PCR and screening cDNA libraries were accomplished as described for α lb. The upstream and downstream PCR primers used were from the Tm3-Tm4 loop and the Tm5-Tm6 loop, respectively: from the sense strand (nucleotide 403-425), 5' CCAACCATCGTCACCCAGAGGAG 3', and from the antisense strand (nucleotide 775-802), 5' TCTCCCGGG AGAACTTGAGGAGCCTCAC 3' (see Figures 3A-3G). The internal probe was: from the sense strand (nucleotide 711-745), 5' TCCGCATCCATCGGAAAAACGCCCCGGCAGGAGGC AGCGGGATGG 3', and from the anti-sense strand (nucleotide 726-771), 5' GAAGTGCGTCTTGGTCTTGGCGCT GGCCATCCCGCTGCCTCCTGCC 3' (see Figures 3A-3G). PCR, library screen, plaque purification excision-rescue from λ Zap II, restriction digestions and sequencing were accomplished as described above for α la.

Expression

α la: The entire coding region of α la (1719 bp), including 150 basepairs of 5' untranslated sequence (5' UT) and 300 bp of 3' untranslated sequence (3' UT), was cloned into the BamHI and ClaI sites of the polylinker-modified eukaryotic expression vector pCEXV-3 (13), called EXJ.HR (unpublished data). The construct involved the ligation of partial overlapping human lymphocyte genomic and hippocampal cDNA clones: 5' sequences were contained on a 1.2 kb SmaI-XhoI genomic

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fragment (the vector-derived BamHI site was used for subcloning instead of the internal insert-derived SmaI site) and 3' sequences were contained on an 1.3 kb XhoI-ClaI cDNA fragment (the ClaI site was from the vector polylinker). Stable cell lines were obtained by cotransfection with the plasmid $\alpha 1a$ /EXJ (expression vector containing the $\alpha 1a$ receptor gene) and the plasmid pGCCos3neo (plasmid containing the aminoglycoside transferase gene) into LM(tk⁻), CHO, NIH3T3 cells, and 293 cells using calcium phosphate technique. The cells were grown, in a controlled environment (37°C., 5% CO₂), as monolayers in Dulbecco's modified Eagle's Medium (GIBCO, Grand Island, NY) containing 25mM glucose and supplemented with 10% bovine calf serum, 100 units/ml penicillin G, and 100 μ g/ml streptomycin sulfate. Stable clones were then selected for resistance to the antibiotic G-418 (1 mg/ml) as described previously (26) and membranes were harvested and assayed for their ability to bind [³H]prazosin as described below (see "Radioligand Binding Assays").

$\alpha 1b$: The entire coding region of $\alpha 1b$ (1563 bp), including 200 basepairs of 5' untranslated sequence (5' UT) and 600 bp of 3' untranslated sequence (3' UT), was cloned into the EcoRI site of pCEXV-3 eukaryotic expression vector (13). The construct involved ligating the full-length containing EcoRI brainstem cDNA fragment from λ Zap II into the expression vector. Stable cell lines were selected as described above.

$\alpha 1c$: The entire coding region of $\alpha 1c$ (1401 bp), including 400 basepairs of 5' untranslated sequence (5' UT) and 200 bp of 3' untranslated sequence (3' UT), was cloned into the KpnI site of the polylinker-modified pCEXV-3-derived (13) eukaryotic expression vector, EXJ.RH (unpublished data). The construct involved

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ligating three partial overlapping fragments: a 5' 0.6kb HincII genomic clone, a central 1.8 EcoRI hippocampal cDNA clone, and a 3' 0.6kb PstI genomic clone. The hippocampal cDNA fragment overlaps with the 5' and 3' genomic clones so that the HincII and PstI sites at the 5' and 3' ends of the cDNA clones, respectively, were utilized for ligation. This full-length clone was cloned into the KpnI sites of the fragment, derived from vector (ie pBluescript) and 3' untranslated sequences, respectively. Stable cell lines were selected as described above.

Radioligand Binding Assays

Transfected cells from culture flasks were scraped into 5ml of 5mM tris-HCl, 5mM EDTA, pH 7.5, and lysed by sonication. The cell lysates were centrifuged at 1000 rpm for 5 min at 4°C. The pellet was suspended in 50mM Tris-HCl, 1mM MgCl₂, and 0.1% ascorbic acid at pH 7.5. Binding of the $\alpha 1$ antagonist [³H]prazosin (0.5 nM, specific activity 76.2 Ci/mmol) to membrane preparations of LM(tk-) cells was done in a final volume of 0.25 ml and incubated at 37°C for 20 min. Nonspecific binding was determined in the presence of 10 μ M phentolamine. The reaction was stopped by filtration through GF/B filters using a cell harvester. Data were analyzed by a computerized non-linear regression program.

Measurement of [³H]Inositol Phosphates (IP) Formation

Cells were suspended in Dulbecco's phosphate buffered saline (PBS), and incubated with 5 μ Ci/ml [³H]m-inositol for 60 min at 37°C, the reaction was stopped by adding CHCl₃:Methanol: HCl (2/1/0.01 v/v). Total [³H]IP were separated by ion exchange chromatography and quantified as described by Forray and El-Fakahany (7).

Calcium Measurements

Intracellular calcium levels ([Ca²⁺]_i) were determined

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with the calcium-sensitive dye fura-2, and microspectrofluorometry, essentially as previously described (1,3). Briefly, cells were plated into polylysine-coated coverslip bottom dishes (MatTek Corporation, Ashland MA). To load with fura-2, cells were washed 3x with HEPES-buffered saline (HBS, in mM: HEPES, 20; NaCl, 150; KCl, 5; CaCl_2 , 1; MgCl_2 , 1; glucose, 10; pH 7.4) and incubated for 30 minutes at room temperature with fura-2 loading solution (5uM fura-2/AM, 0.03% pluronic F-127, and 2% heat-inactivated fetal calf serum, in HBS). After loading, cells were washed 3x with HBS, 1ml of HBS was added, and the dish was placed on the microscope for determination of $[\text{Ca}^{2+}]_i$. $[\text{Ca}^{2+}]_i$ was measured with a Leitz Fluovert microscope equipped for UV-transmission epifluorescence. Fura-2 fluorescence was alternately excited at 340 and 380nm (0.25 sec), and a pair of readings (500nm long pass) was taken every two seconds, and recorded by a personal computer interfaced to a data acquisition and control unit from Kinetek (Yonkers, NY). To determine $[\text{Ca}^{2+}]_i$ from the experimental data the background fluorescence was subtracted, and the corrected ratios were converted to $[\text{Ca}^{2+}]_i$ by comparison with buffers containing saturating and low free calcium, assuming a K_d of 400 nM (3).

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RESULTS

α 1a: We screened a human genomic lymphocyte library with a rat PCR fragment that exhibited homology with the α 1-AR family. A total of six clones were isolated and characterized by Southern blot analysis. One clone, h13, contained a 4.0kb XbaI fragment which hybridized with the radiolabeled rat PCR fragment and was subsequently subcloned into pUC vector. DNA sequence analysis indicated greatest homology to human α 1a and rat α 1a ARs. This clone contained the initiating methionine through Tm6 with \approx 1.0-1.5kb 5' UT region. Subsequent Southern blot, analysis, subcloning and sequencing analysis indicated the presence of a SmaI site \approx 150nts. 5' to the initiating methionine codon. The homology between h13 and rat α 1a adrenergic gene breaks just downstream of Tm6, indicating an intron which is located in an analogous region in the α 1b- and α 1c-AR genes (4,20). In order to obtain a full-length clone, aliquots of human cDNA libraries totaling $\approx 1.5 \times 10^6$ recombinants was screened by polymerase chain reaction using specific oligonucleotide primers from sequence determined off the genomic clone (see Materials and Methods). A positive- containing human hippocampal cDNA library (Stratagene, LaJolla, CA.) in λ Zap II ($\approx 1.5 \times 10^6$ recombinants) was screened using traditional plaque hybridization with an internal probe (see Materials and Methods) and resulted in the isolation of two positive cDNA clones, one containing the upstream sequences (from 5' UT through the 5-6 loop; hH22) and the other containing downstream sequences (from within Tm5 through \approx 200 nts. with a common XhoI site being present within this common region.

The complete full-length gene was constructed by splicing together two restriction fragments, one being the 3' cDNA (hH14) and the other being the 5' genomic

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clone (h13), using a unique restriction site (XhoI) present in the overlapping region. In addition, another construct was accomplished by ligating the two cDNA clones (hH14 and hH22), using the overlapping XhoI site; however, since this construct produced the same pharmacology as the genomic/cDNA construct, we will not discuss this recombinant (unpublished observation). The genomic/cDNA construct contains an open reading frame of 1719 bp and encoding a protein of 572 aa in length, having a relative molecular mass of $\approx 63,000$ daltons. Hydropathy analysis of the protein is consistent with a putative topography of seven transmembrane domains, indicative of the G protein-coupled receptor family. Initial sequence analysis revealed that clone $\alpha 1a$ /EXJ was most related to an AR since it contained a number of conserved structural features/residues found among the members of the adrenergic receptor family, including conserve cysteines in the second and third extracellular loops, a conserved glycine residue in Tm1, aspartic acid residues in Tm regions II and III, conserved valine residues in TmIII, the DRY sequence at the end of TmIII, the conserved proline residues of Tm regions II, IV, V, VI and VII, and the consensus D-V-L-X-X-T-X-S-I-X-X-L-C IN Tm3 and the consensus G-Y-X-N-S-X-X-N-P-X-I-Y in the Tm VII, both consensus unique to the adrenergic receptor family (2,26). Other features of this human $\alpha 1a$ receptor gene are the presence of two potential sites for N-linked glycosylation in the amino terminus (asparagine residues 65 and 82; Figures 1a-1I) and the presence of several serines and threonines in the carboxyl terminus and intracellular loops, which may serve as sites for potential phosphorylation by protein kinases.

$\alpha 1b$: We screened a human genomic placenta library with probes derived from Tm3, 5 and 6 regions of serotonin

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5HT1D_B under low stringency. Out of several hundred positive clones pursued by Southern blot analysis, subcloning and sequencing, one resembled the α_1 adrenergic family of receptors. This genomic fragment contained Tm3 through Tm6 of a receptor which was most closely related to rat and hamster α_{1b} receptors. In order to obtain a full-length clone, several human cDNA libraries were screened by PCR using primers derived from the 5-6 loop region of the genomic clone (see Materials and Methods). A positive-containing human brainstem cDNA library (Stratagene, LaJolla, CA) in λ ZAPII ($\approx 2 \times 10^6$ recombinants) was screened using traditional plaque hybridization with an internal probe, resulting in the isolation of two identical cDNA clones, containing an insert size of 2.4 kb. Upon sequencing, this clone was found to contain the initiating MET aa, Tm1 through Tm7, and 5' and 3' UT sequences, suggesting a full-length clone on a single EcoRI fragment. This cDNA clone contains an open reading frame of 1563 bp and encodes a protein of 520 aa in length, having a relative molecular mass of $\approx 57,000$ daltons. Hydropathy analysis of the protein is consistent with a putative topography of seven transmembrane domains, indicative of the G protein-coupled receptor family.

Sequence analysis revealed that clone α_{1b} /pCEXV was most related to adrenergic receptor since it contained a number of conserved structural features found among the adrenergic receptor family, as described for α_{1a} receptor (see above). This human α_{1b} receptor contains potential sites for N-linked glycosylation in the amino terminus (asparagine residues 10, 24, 29, 34 in Fig. 2A-2H), consistent with the finding that the α_1 AR is glycosylated (4,19).

α_{1c} : We screened a human genomic lymphocyte library

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with probes derived from the third, fifth and sixth transmembrane regions of serotonin 5HT1A under low stringency. Out of several hundred positive clones analyzed by Southern blot analysis, subcloning and sequencing (see Materials and Methods), one phage clone resembled a novel α_1 AR. This genomic fragment contained Tm1 through Tm6 of a receptor with high homology to the bovine α_{1c} receptor and thus suggesting the presence of an intron downstream of Tm6, as shown for the α_1 receptor family (4,12,20). In order to obtain a full-length clone, several human cDNA libraries were screened by PCR, as described for α_{1b} (also see Materials and Methods). A positive-containing human hippocampal cDNA library (Stratagene, LaJolla, CA) in λ ZAPII ($\approx 2 \times 10^6$ recombinants) was screened, as described for α_{1b} . A positive clone (hH 20) was identified which contained a 1.7kb EcoRI cDNA fragment insert. However, this cDNA clone lacked both the amino end of the receptor (the 5' end of the clone terminated at the 5' end of Tm2) and part of the carboxyl tail (the 3' end of the clone corresponded to 40 aa upstream from the "putative" stop codon). Since an alternative genomic subclone which contained the initiating MET codon in addition to Tm1 through Tm6 was available, we needed to obtain the complete 3' carboxyl tail in order to complete the construct of the full-length clone. This was accomplished by using overlapping 45-mer oligonucleotide primers (corresponding to nts. 1142-1212 in Fig. 3), designed within the carboxyl tail of the receptor (at the 3' end of the hH20 cDNA clone), to screen a human lymphocyte genomic library in order to isolate a genomic clone containing the carboxyl tail that includes the termination codon. Two identical positive human lymphocyte genomic clones were isolated from this library. A 0.6 kb PstI fragment was subcloned and shown to contain most of the carboxyl tail (≈ 20 aa downstream

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of Tm7) through the termination codon and ≈ 200 bp of 3' UT sequence.

5 The complete full-length gene was constructed by
splicing together three restriction fragments: A 0.6 kb
HincII fragment from the genomic clone, containing ≈ 0.4
kb of 5' UT sequence and the initiating MET codon
through Tm2; the 0.8 kb HincII-PstI fragment from the
10 hH cDNA clone, which contains Tm2 through part of the
carboxyl tail, overlapping with the 5' genomic clone by
20 nts. (sharing the unique HincII site at position 196
in Fig.3); and a 0.6 kb PstI fragment from the second
h1 genomic clone, which contains the carboxyl tail, the
stop codon and ≈ 0.2 kb of 3' UT sequence, and
15 overlapping with the hH cDNA clone (sharing the unique
Pst I site within the carboxyl tail at position 1038 in
Figures 3A-3G).

20 The resulting genomic/cDNA/genomic construct contains
an open reading frame of 1401 bp and encoding a protein
of 466 aa in length, having a molecular weight of
 $\approx 51,000$ daltons. Hydropathy analysis of the protein is
consistent with a putative topography of seven
transmembrane domains, as indicated for the previously
25 described human α_{1a} and α_{1b} receptors and indicative of
the G protein-coupled receptor family. Sequence
analysis revealed that clone α_{1c} /EXJ was most related to
adrenergic receptor because it contained the structural
features commonly found among the adrenergic receptor
30 family of receptors, as described for the α_{1a} receptor
above. Other features of this human α_{1c} receptor gene
is the presence of three potential sites for N-linked
glycosylation in the amino terminus, at the same
position described for the bovine α_{1c} receptor
35 (asparagine residues 7, 13 and 22 in Figure 3A-3G) (20).
Several threonines and serines exist in the second and
third cytoplasmic loops of this α_{1c} receptor, which may

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serve as potential sites for protein kinases and phosphorylation.

Table 1. Competition of adrenergic agonists and antagonists for the binding of [³H]prazosin to membranes prepared from LM(tk⁻) cells expressing the human α_{1a} , α_{1b} , and α_{1c} -adrenergic receptor cDNA. Membrane preparations from stably transfected cell lines increasing concentrations of various agonists or antagonists as described under "Materials and Methods". Data is shown as the mean \pm S.E.M. of the binding parameters estimated by a computerized non-linear regression analysis obtained in three independent experiments each performed in triplicate.

	pKi		
	α_{1a}	α_{1b}	α_{1c}
AGONISTS			
Norepinephrine	6.633 \pm 0.12	5.614 \pm 0.09	5.747 \pm 0.18
Epinephrine	6.245 \pm 0.10	5.297 \pm 0.15	5.511 \pm 0.13
Oxymetazoline	5.903 \pm 0.16	5.919 \pm 0.07	7.691 \pm 0.10
Naphazoline	6.647 \pm 0.18	6.155 \pm 0.04	6.705 \pm 0.22
Xylometazoline	5.913 \pm 0.20	6.096 \pm 0.30	7.499 \pm 0.19
ANTAGONISTS			
Prazosin	9.479 \pm 0.19	9.260 \pm 0.23	9.234 \pm 0.13
WB-4101	8.828 \pm 0.12	7.909 \pm 0.13	9.080 \pm 0.09
(+) Niguldipine	6.643 \pm 0.10	6.937 \pm 0.12	8.693 \pm 0.18
Indoramin	6.629 \pm 0.09	7.347 \pm 0.17	8.341 \pm 0.25
5-Methyl Urapidil	7.795 \pm 0.15	6.603 \pm 0.09	8.160 \pm 0.11
HEAT	7.857 \pm 0.13	8.474 \pm 0.10	8.617 \pm 0.10
Urapidil	6.509 \pm 0.18	5.932 \pm 0.11	6.987 \pm 0.14

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Rauwolscine	5.274 ± 0.12	4.852 ± 0.08	4.527 ± 0.11
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Pharmacological Analysis: To further assess the functional identity of the cloned cDNA the coding regions were subcloned into the pCEXV-3 expression vector, and LM(tk-) cell lines stably expressing the human cDNA encoding each of the three α_1 -ARs were established. Membrane preparations of these cell lines showed high affinity binding of [3 H]prazosin, with Kd values of 0.21 ± 0.03 nM (Bmax= 0.72 ± 0.04 pmol/mg prot), 0.88 ± 0.1 nM (Bmax= 4.59 ± 0.21 pmol/mg prot) and 0.39 ± 0.08 nM (Bmax= 1.9 ± 0.04 pmol/mg prot) for the cells expressing the α_{1a} , α_{1b} , and α_{1c} -ARs respectively. In contrast in competition binding experiments rauwolscine showed extremely low affinity at the three cloned receptors (Table 1), consistent with their identity as α_1 -AR. The α -adrenergic agonists NE and epinephrine were found to be 6 and 5-fold respectively, more potent at the human α_{1a} -AR, conversely the imidazoline derivatives such as oxymetazoline and xylometazoline showed 52-fold higher potency at the α_{1c} -AR. Similarly, several antagonists showed marked differences in their potency to inhibit [3 H]prazosin binding from the cloned human α_1 receptors subtypes. The selective antagonists WB-4101 and 5-methyl-urapidil showed high affinity for the human α_{1c} subtype (0.8 and 7 nM respectively), followed by less than 2-fold lower potency at the human α_{1a} and at least an order of magnitude (15 and 36-fold respectively) lower potency at the human α_{1b} -AR. Similarly, indoramin was 50 and 10-fold more potent at the α_{1c} than at the α_{1a} and α_{1b} respectively. The calcium channel blocker (+)-niguldipine showed the highest selectivity for the three α_1 -AR subtypes, displacing [3 H]prazosin 112 and 57-fold more potently from the α_{1c} than from α_{1a} and α_{1b} transfected cells respectively.

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Table 2. Receptor-mediated formation of [^3H]IP in cell lines transfected with the human α_1 -adrenergic receptors cDNA.

Cell lines stably expressing the human α_1 -adrenergic receptors were obtained and the IP formation was measured in the absence or presence of $10\mu\text{M}$ norepinephrine (NE) in the presence of 10 mM LiCl as described under "Material and Methods". Data are shown as mean \pm S.E.M. of three independent experiments performed in triplicate.

	Cell Line	[^3H]IP	Fold Stimulation	Receptor
		dpm/dish		^a Density
				pmol/mg Prot
15	293 α_{1a}			3.30
	Control	288 \pm 29		
	NE	3646 \pm 144	13	
	CHO α_{1b}			0.49
20	Control	1069 \pm 26		
	NE	5934 \pm 309	6	
	NIH3T3 α_{1c}			0.24
	Control	722 \pm 61		
25	NE	13929 \pm 1226	19	

^a Determined by [^3H]Prazosin binding.

The formation of [^3H]IP was measured in 293, CHO, and

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NIH3T3 cell stably expressing the cloned human α_{1a} , α_{1b} , α_{1c} -ARs respectively, to assess the functional coupling of these receptors with the activation of phosphatidylinositol specific phospholipase C (PI-PLC). As shown in Table 2, the adrenergic agonist NE (10 μ M) activated the formation of IP by 13-fold in cells expressing the α_{1a} receptor, and by 5 and 15-fold in cells expressing the α_{1a} , α_{1b} and α_{1c} receptors respectively. Furthermore, when cells expressing α_{1b} and α_{1c} receptors were incubated in the presence of 10 μ M NE, a rapid increase of cytosolic calcium was observed. The response was characterized by an early peak, followed by a plateau that slowly declined towards resting calcium levels (Fig 7). The concentration of $[Ca^{2+}]_i$ was increased by 172 \pm 33 (n=6), 170 \pm 48 (n=6) and 224 \pm 79 nM (n=6) in cell lines transfected with the α_{1a} , α_{1b} and α_{1c} receptors respectively. The changes in $[Ca^{2+}]_i$ induced by NE were suppressed by preincubation of the cells with 10 nM prazosin, indicating that the calcium response was mediated by α_1 -ARs.

We have cloned DNA representing three α_1 -ARs subtypes (α_{1a} , α_{1b} and α_{1c}) from human brain cDNA and genomic DNA. Of all known G protein-coupled receptor sequences (EMBL/Genbank Data Base), the greatest homology was found between α_{1a} /EXJ and the rat α_{1a} AR (12), rat α_{1d} AR (16) and a previously reported putative human " α_{1a} " adrenergic receptor (H318/3) (2). Comparison of the human α_{1a} deduced aa sequence with known α_{1a} ARs indicates the greatest concentration of identical aa to be in the transmembrane domains. In these Tm regions, the percentage of identity for the human α_{1a} AR is 98% compared to rat α_{1a} AR (12) (this is approximately the same for rat α_{1d} since rat α_{1d} AR is the same as rat α_{1a} AR, except for two amino acid differences), 100% with the previously reported H318/3, 78% with the human α_{1b} receptor (see below), and 69% with the human α_{1c}

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receptor (see below), which is typical among subtypes. When considering the full-length proteins, the percent identity drops and is only 50% for the human α_{1b} and 49% for the human α_{1c} receptor. Both the alignment (see
5 Fig. 4) and percent identity of this human α_{1a} sequence, relative to other members of the AR family strongly suggest that this is a new receptor and is the human species homolog of the rat α_{1a} receptor.

10 Figure 4 shows a comparison between the deduced aa sequence of α_{1a} /EXJ and the sequences of rat α_{1a} and HAR. An overall homology of 83.5% aa identity with rat α_{1a} and 86.5% aa identity with the previously published H318/3 clone was observed, suggesting that our human α_{1a}
15 receptor is not any more related to the previously published putative human " α_{1a} " than it is to the rat α_{1a} receptor. In fact, in support of this conclusion, is the fact that the overall aa homology of rat α_{1a} receptor with our human α_{1a} receptor is 83.5% but is
20 only 72% compared to the H318/3 receptor. The main differences between our human α_{1a} receptor and the previously reported " α_{1a} " receptor in relation to the rat α_{1a} are indicated in Fig. 4. Most notably are the differences observed at both the amino and carboxyl
25 ends of the receptor. Specifically, both our human α_{1a} and rat α_{1a} use the starting MET aa at position 1 (see Fig. 4) whereas the previously published H318/3 uses the starting MET 48 aa downstream. Also, the amino terminus of the H318/3 clone is completely divergent
30 from either rat α_{1a} or our human α_{1a} receptor until about 12 aa upstream of Tm1 where significant homology begins. Similarly, in the carboxyl tail, the homology of H318/3 diverges \approx 90 aa upstream from the stop codon of either rat or our human α_{1a} receptor and instead,
35 uses a stop codon 30 aa upstream from the stop codon on either of these receptors. Finally, the H318/3 clone has an amino terminal extracellular region that does

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not contain potential sites for N-linked glycosylation (2), in contrast to the rat α_{1a} or our human α_{1a} receptor, which contains two potential sites (12, see also Fig. 1 and above). Thus, these data strongly suggest that our human α_{1a} receptor is different in sequence from the previously reported putative human " α_{1a} " (H318/3) but is more related to the previously published rat α_{1a} receptor. Interestingly, the rat α_{1a} aa sequence diverges from both human α_{1a} receptors for ≈ 65 aa in the carboxyl tail (position 434-508 in Fig. 1); however, homology is seen again in our human α_{1a} receptor but not with H318/3, downstream from this region.

The cloning of different α_1 receptor subtypes permits analysis of both the pharmacological and functional properties of adrenergic receptors. The human α_{1b} /pcEXV clone exhibited the greatest homology with the rat and hamster α_{1b} receptors, out of all known G protein-coupled receptor clones (EMBL/Genbank Data Bank). Comparison of the human α_{1b} deduced aa sequence with known α_1 ARs indicates the greatest homology in the transmembrane regions. In these Tm regions, the percent identity for the human α_{1b} AR is 99% compared to either rat (25) or hamster (4) α_b receptor, 78% with human α_{1a} receptor and 75% with human α_{1c} receptor, which is typical among subtypes. When analyzing the full-length proteins, the percent identity slightly drops and is 94.5% compared to rat α_{1b} , 95.5% compared to hamster α_{1b} receptor, 50% compared to human α_{1a} and 51% compared to human α_{1c} receptor. Both the alignment (see Fig. 5) and percent identity of this human α_{1b} sequence, relative to other members of the AR family, strongly suggest that this clone represents a new receptor and is the human species homologue of the rat/hamster α_{1b} receptor. Figure 5 shows a comparison between the deduced amino acid sequence of α_{1b} /pcEXV and

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the aa sequence of rat α_{1b} and hamster α_{1b} receptors.

A third human adrenergic receptor clone, α_{1c} /EXJ, showed the greatest homology with the bovine α_{1c} AR gene (20),
5 from all known G protein-coupled receptor sequences (EMBL/Genbank Data Bank). Comparison of the human α_{1c} deduced aa sequence with the α_1 ARs indicates the greatest homology to be in the transmembrane regions. In these Tm regions, the percent identity for the human
10 α_{1c} AR is 97% compared to the bovine α_{1c} AR (20), 75% with human α_{1b} receptor and 69% with human α_{1a} receptor, which is typical among subtypes. When one examines the full-length proteins, the percent identity drops and is only 51% compared to either the human α_{1b} or human α_{1a}
15 receptor. Figure 6 shows a comparison between the deduced amino acid sequence of α_{1c} /EXJ and the aa sequence of bovine α_{1c} . An overall homology of 92% aa identity with bovine α_{1c} receptor was observed. Both the alignment (see Fig. 6) and percent identity of this
20 human α_{1c} sequence, relative to other members of the AR family, strongly suggest that this clone represents a new receptor and is the human species homologue of the bovine α_{1c} receptor.

25 The stable expression of the three cloned human α_1 receptors enabled the characterization of their pharmacological as well as their functional properties and allowed identification of certain unique features of the human receptors, not predicted from previous
30 data. The rank-order of potency of known α -adrenergic agonists and antagonists to compete with [3 H]prazosin in binding assays, confirmed that the cloned cDNAs encode three human receptors of the α_1 -AR family. Moreover, the potencies of selective antagonists such
35 as WB-4101 and 5-methyl-urapidil at the three human α_1 -receptors were found to be in close agreement with the potencies of these antagonists at the cloned rat α_{1a} ,

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hamster α_{1b} , and bovine α_{1c} (4, 12, 20). These results suggest that the sequence homology between the three mammalian α_1 receptors resulted in a conservation of their pharmacological properties across different species. In the past the pharmacological characterization of α_1 -adrenergic receptors took advantage of the existence of selective antagonists such as WB-4101 and 5-methyl-urapidil that bind with high affinity to a subset of α_1 -receptors classified as α_{1a} (9, 15). Our results using these selective antagonists indicate that these antagonists bind with similar affinity to both human α_{1a} and α_{1c} -receptors, and that they can only discriminate between either of these two subtypes and the α_{1b} receptor. The calcium channel blocker (+)-niguldipine was found to bind with high affinity to a subset of α_1 -receptors also labeled by [3 H]5-methyl-urapidil in rat brain, thus defining this antagonist as α_{1a} selective (8). The high affinity of the human α_{1c} receptor for (+)-niguldipine and the fact that it binds to the human α_{1a} and α_{1b} subtypes, with at least an order of magnitude lower affinity, strongly supports the notion that the human α_{1c} gene encodes the pharmacological α_{1a} -receptor subtype. The possibility that this also holds true in the rat, is suggested by the fact that the potency of (+)-niguldipine for the rat α_{1a} clone is also at least an order of magnitude lower than that found for this antagonist in rat tissues. Moreover in spite of the earlier reports on the absence of the bovine α_{1c} cognate in rat tissues (20), (24,21) pharmacological evidence suggests that this species express an α_1 receptor similar to the cloned α_{1c} receptor. These data altogether indicate that in trying to match the pharmacological subclassification of the α_1 -ARs with the evidence from molecular cloning studies, the initial assignment of the cloned rat α_{1a} receptor with the α_{1a} receptor subtype was inadequate. Recently, a rat

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5 cDNA clone 99.8% homologous to the rat α_{1a} -receptor, was described as a novel α_{1d} subtype (16); however, this incorrect classification was due to the poor correlation between the affinities of α_{1a} -selective antagonists in tissue preparations versus the cloned rat α_{1a} receptor.

10 The three human α_1 receptor subtypes were able to induce the formation of IP, consistent with the known functional coupling of α_1 -ARs, through a GTP-dependent protein to the activation of PI-PLC. In addition we demonstrated that upon receptor activation by adrenergic agonists, the human α_1 subtypes induced transient changes three in $[Ca^{2+}]_i$. Consistent with the
15 mobilization of calcium from intracellular stores by inositol-1,3,5 triphosphate, released by the receptor-mediated activation of PI-PLC.

20 We have cloned and expressed three human cDNA that encode functional α_1 -ARs. These three transcripts display significant pharmacologic as well as molecular features to constitute distinct α_1 -AR subtypes. In sharp contrast with the restricted expression of the rat and bovine transcripts, our findings indicate that
25 species homologs of the three α_1 -ARs are expressed in human tissues. These findings together with recent reports on the dissimilar tissue distribution of the α_{1b} and α_{1c} receptor cognates between animal species such as rat and rabbit (21), commonly used in the development
30 of novel α_1 -adrenergic agents, emphasize the need to study the pharmacological properties of the human α_1 -receptors. In this regard, the results from this study on the selectivity of clinically effective antihypertensives such as indoramin, as well as
35 vasoconstrictors such as oxymetazoline and xylometazoline for the human α_{1c} -AR, suggest a potential role for this α_1 -receptor subtype in the physiological

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control of vascular tone in the human. Thus, the availability of cell lines expressing each of the human α_1 -receptor subtypes constitute a unique tool in the design of subtype specific agonists and antagonists, that can be targeted to selective therapeutic applications. Of specific interest for therapeutics are subtype selective alpha-1 antagonists for the treatment of Benign Prostatic Hypertrophy, coronary heart disease, insulin resistance, atherosclerosis, sympathetic dystrophy syndrome, glaucoma, cardiac arrhythmias, erectile dysfunction, Reynaud's syndrome, hypertension and urinary retention (44,27,31,32,33,34,35,48). Further interest exists for subtype selective alpha-1 agonists for the treatment of congestive heart failure, nasal congestion, urinary incontinence and hypotension(45,46,47,48). In each case, a more selective drug is expected to reduce the side effects which presently limit this avenue of therapy.

The following compounds were synthesized in order to evaluate their ability to act as antagonists of α_1 -receptor function in human prostate. The synthetic methods used to synthesize are provided herein.

The following Experimental Details are set forth to aid in an understanding of the invention, and are not intended, and should not be construed, to limit in any way the invention set forth in the claims which follow thereafter.

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Experimental Details.

Prazosin and 5-methylurapidil were obtained from Research Biochemicals, Inc. A30360 (4-fluoro-4-(8-fluoro-1,3,4,5-tetrahydro-2H-pyrido[4,3-b]indol-2-yl)butyrophenone hydrochloride) was obtained from Aldrich Chemical Co. Other compounds were prepared according to the examples which follow.

Example 1**Synthesis of Terazosin Hydrochloride****N-(2-Furoyl)piperazine**

This compound and its preparation has been described in Great Britain Patents 1,390,014 and 1,390,015.

Piperazine hexahydrate (194 g, 1 mole) was dissolved in 250 ml H₂O. The solution was acidified to pH 4.5 with 6 N HCl. Furoyl chloride (130.5 g, 1 mole, Aldrich) was added along with 10% NaOH solution at such a rate that the pH was maintained at 4.5. After 1 hour, the solution was made basic (pH = 8.5) with NaOH solution. The reaction mixture was continuously extracted with chloroform for 36 hours. The CHCl₃ extract was dried over MgSO₄, and filtered. Distillation gave 108.2 g product (60%), b.p. 132° - 138° C/0.6 mm Hg, m.p. 69° - 70 °C.

N-(Tetrahydro-2-furoyl)piperazine

The furoylpiperazine of Example 1 was converted to the hydrobromide salt (m.p. 173° - 175° C). This salt (39.0 g) in 250 ml methyl alcohol and 9.0 g Raney nickel was hydrogenated at 3 atm. After uptake of H₂ ceased, the catalyst was filtered, the solvent concentrated, and the residue crystallized from isopropyl alcohol to give 35.2 g. tetrahydrofuroylpiperazine HBr, m.p. 152° - 156 °C. This was suspended in 20 ml H₂O. Then 10.5 g 50%, NaOH solution was added slowly followed by 2.0 g solid Na₂CO₃. This was extracted with 4 x 100 ml portions of

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warm CHCl_3 . The CHCl_3 extractions were distilled to give 22.5 g tetrahydrofurolylpiperazine, b.p. $120^\circ - 125^\circ\text{C}/0.2 \text{ mm Hg}$.

5 **2[4-(Tetrahydro-2-furoyl)piperazinyl]-4-amino-6,7-dimethoxyquinazoline hydrochloride**

To 7.00 g 2-chloro-4-amino-6,7-dimethoxyquinazoline (Lancaster Synthesis) in 50 ml methoxyethanol was added 10.8 g, tetrahydrofurolylpiperazine, and the mixture
10 refluxed 3 hours. The clear solution was concentrated and an aqueous solution of potassium bicarbonate was added. The resultant solid that formed was filtered and washed with water. It was then added to methanol and the resulting suspension was acidified with a
15 solution of hydrogen chloride in isopropyl alcohol. The resulting solution was concentrated and the residue crystallized from isopropyl alcohol giving 8.12 g. of product, m.p. $278^\circ - 279^\circ\text{C}$.

20

Example 2

Preparation of Indoramin

4-Benzamido-1-[2-(3-indolyl)ethyl]pyridinium Bromide

A solution of 4-benzamidopyridine (1.98 g) and 3-(2-bromoethyl)indole (2.24 g) in EtOH (15 ml) was refluxed
25 for 2 hours, and the crystallized product (3.13 g, mp $264 - 266^\circ\text{C}$) was collected by filtration from the hot reaction mixture. Recrystallization gave the hydrate.

3-[2-4-Benzamidopiperid-1-yl]ethyl]indole (Indoramin)

30

4-Benzamido-1-[2-(3-indolyl)ethyl]pyridinium bromide (3.0g) in 91% EtOH (300 ml) containing Et_3N (0.8 g) was hydrogenated in the presence of freshly prepared W-7 Raney Ni catalyst (ca. 3 g) at 28.12 kg/cm^2 and 50° for
35 4 hours. After filtering off the catalyst, the filtrate was evaporated and the residue was shaken with CHCl_3 and 2 N NaOH. The resulting insoluble material

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(1.61 g, mp 203 - 206°C) was collected and dried. Recrystallization from EtOH gave the product (1.34 g), as colorless needles.

5

Example 3

Preparation of 1-(3-benzoylpropyl)-4-benzamidopiperidine (Compound 9)

A mixture of 4-chlorobutyrophenone (447 mg, 2.45 mmol), 4-benzamidopiperidine (500 mg, 2.45 mmol) and K₂CO₃ (338 mg, 2.45 mmol) was heated up in boiling water bath for 1 hour. The reaction mixture was portioned between water and CHCl₃. The organic layer was separated and dried over Na₂SO₄. After filtration and removal of solvent, the residue was purified by chromatography (SiO₂, MeOH:CHCl₃, 5:95). Recrystallization from AcOEt/hexane gave a white powder (78 mg, 8.2%). mp 143-144°C; ¹H NMR (CD₃OD, 400MHz) δ 1.65 (dq, J₁=3.16 Hz, J₂=11.9 Hz, 2H), 1.90-2.00 (m, 4H), 2.18 (t, J=11.9 Hz, 2H), 2.48 (m, 2H), 3.00-3.10 (m, 4H), 3.88 (m, 1H), 7.40-8.00 (m, 10H); Mass spectrum (M+1)⁺ at m/z 351.

20

Example 4

Preparation of 1-[3-(4-chlorobenzoyl)propyl]-4-benzamidopiperidine (Compound 7)

A mixture of 3-(4-chlorobenzoyl)propyl bromide (640 mg, 2.45 mmol), 4-benzamidopiperidine (500 mg, 2.45 mmol) and K₂CO₃ (1.01 g, 7.34 mmol) in 50 ml of acetone was heated up to refluxing condition for 48 hours. The solid was removed by filtration. Concentration of filtrate in vacuo gave a yellowish solid, which was purified by chromatography (SiO₂, MeOH:CHCl₃, 5:95). 320 mg (33.9%) of white powder was obtained ¹H NMR (CDCl₃, 300 MHz) δ 1.46 (dq, J₁=1.0 Hz, J₂=8.4 Hz, 2H), 1.90-2.10 (m, 4H), 2.16 (m, 2H), 2.43 (t, J=6.9 Hz, 2H), 2.80-2.90 (m, 2H), 2.97 (t, J=6.9 Hz, 2H), 3.97 (m, 1H), 5.92 (d, J=7.8 Hz, 1H, N-H), 7.40-8.00 (m, 9H); Product was converted to HCl salt and

35

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recrystallized with MeOH/Et₂O, mp 243-244°C; Calcd for C₂₂H₂₅ClN₂O₂·HCl·H₂O: C 60.15, H 6.37, N 6.37; Found: C 60.18, H 6.34, N 6.29.

5

Example 5**Preparation of SKF-104856****1-[(4-Chlorophenyl)thio]-2-propanone**

Chloroacetone (32.3 g, 0.347 mol) was added to a mixture of 4-chlorothiophenol (50 g, 0.347 mmol) and sodium hydroxide (14 g, 0.347 mol) in water (400 ml) and the mixture was stirred at 25°C for 1 hour. The mixture was extracted with ethyl ether and the organic phase was washed with water, dried with magnesium sulfate and concentrated to give 69 g (99%) of 1-[(4-chlorophenyl)thio]-2-propanone.

5-Chloro-3-methylbenzo(b)thiophene

1-[(4-Chlorophenyl)thio]-2-propanone (50 g, 0.25 mol) was added to polyphosphoric acid (300 g) and the mixture was stirred as the temperature was gradually raised to 120°C as an exotherm started. The mixture was stirred at 130°C for 1 hour, diluted with water, extracted with ethyl ether and the organic phase was dried and concentrated. The residue was stirred in methanol (200 ml), filtered and the filtrate concentrated to give 17.5 g (40%) of 5-chloro-3-methylbenzo(b)thiophene: bp 120°C (0.6 mm Hg).

Ethyl 5-chloro-3-methylbenzo(b)thiophene-2-carboxylate

n-Butyllithium in hexane (2.6 M, 2.3 ml) was added to a solution of 5-chloro-3-methylbenzo(b)thiophene (1.0 g, 6 mmol) in ethyl ether (20 ml) stirred at 0°C under argon. The mixture was stirred for 30 minutes and transferred slowly under argon pressure to a stirred solution of ethyl chloroformate (0.63 g, 6 mmol) in ethyl ether (20 ml). The mixture was stirred at 0°C for 30 minutes and at 25°C for 1.5 hours. The mixture

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was treated with water and the organic phase was dried, concentrated and triturated with hexane to give 1.0 g (67%) of ethyl 5-chloro-3-methylbenzo(b)thiophene-2-carboxylate: mp 92.5 - 94 °C.

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Ethyl 3-bromomethyl-5-chlorobenzo(b)thiophene-2-carboxylate

A mixture of ethyl 5-chloro-3-methylbenzo(b)thiophene-2-carboxylate (9.0 g, 0.035 mol), N-bromosuccinimide (6.53 g, 0.037 mol) and benzoyl peroxide (130 mg) in carbon tetrachloride (150 ml) was refluxed and illuminated with sunlamp for 2 hours. The resulting suspension was cooled, filtered and the filter cake was triturated with methanol to give 9.9 g, (85%) of the methanol-insoluble ethyl 3-bromomethyl-5-chlorobenzo(b)thiophene-2-carboxylate: mp 148-150°C.

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Ethyl 5-Chloro-3-[N-(2,2-dimethoxyethyl)-N-methyl(aminomethyl)]benzo(b)thiophene-2-carboxylate

A mixture of ethyl 3-bromomethyl-5-chlorobenzo(b)thiophene-2-carboxylate (11 g, 0.033 mol), methylaminoacetaldehyde dimethyl acetal (4.76 g, 0.04 mol) and potassium carbonate (11.4 g, 0.8 mol) in dry acetone (200 ml) was stirred for 48 hours, filtered and the filtrate concentrated to give 11.8 g, (96%) of ethyl 5-chloro-3-(N-2,2-dimethoxyethyl)-N-methyl(aminomethyl)benzo(b)thiophene-2-carboxylate.

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Ethyl 7-chloro-3,4-dihydro-4-methylthieno[4,3,2-ef]-[3]benzazepine-2-carboxylate

Ethyl 5-chloro-3-[N-(2,2-dimethoxyethyl)-N-methyl(aminomethyl)]benzo(b)thiophene-2-carboxylate (3.0 g, 8.1 mmol) was added in portions to trifluoromethanesulfonic acid (10 ml) stirred at 0°C under argon. The mixture was stirred at 25°C for 45 minutes and diluted with water. The mixture was basified with aqueous sodium hydroxide and extracted

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with ethyl ether to give ethyl 7-chloro-3,4-dihydro-4-methylthieno-[4,3,2-ef][3]benzazepine-2-carboxylate.

5 **Ethyl 7-chloro-3,4,5,6-tetrahydro-4-methylthieno[4,3,2-ef][3]benzazepine-2-carboxylate**

Diborane in tetrahydrofuran (1 M, 40 ml) was added to a solution of ethyl 7-chloro-3,4-dihydro-4-methylthieno[4,3,2-ef][3]benzazepine-2-carboxylate (2.8 g) in tetrahydrofuran (30 ml) stirred at 0°C. The mixture was refluxed for 3 hours and stirred at 25°C for 18 hours, cooled, treated with methanol (50 ml), refluxed for 18 hours and concentrated. The residue was triturated with ethyl ether-hexane (3:1) to give 1.6 g (84%) of ethyl 7-chloro-3,4,5,6-tetrahydro-4-methylthieno[4,3,2-ef][3]benzazepine-2-carboxylate: mp 138-140 °C. The free base was treated with hydrogen chloride to give ethyl 7-chloro-3,4,5,6-tetrahydro-4-methylthieno[4,3,2-ef][3]benzazepine-2-carboxylate hydrochloride: mp 240°C.

20 **7-Chloro-3,4,5,6-tetrahydro-4-methylthieno[4,3,2-ef][3]benzazepine-2-methanol**

A solution of ethyl 7-chloro-3,4,5,6-tetrahydro-4-methylthieno[4.3.2-ef][3]benzazepine-2-carboxylate (4.0 g, 12.9 mmol), in ethyl ether (48 ml) was treated with lithium aluminum hydride (0.53 g, 14 mmol). The mixture was stirred for 1.5 hours, cooled and treated carefully with water (2.0 ml), 10% sodium hydroxide (1.0 ml) and water (2.0 ml). The resulting mixture was filtered and the solvent evaporated to give 1.9 g (57%) of 7-chloro-3,4,5,6-tetrahydro-4-methylthieno[4,3,2-ef][3]benzazepine-2-methanol: mp 184-185°C.

35 **7-Chloro-3,4,5,6-tetrahydro-4-methylthieno-4,3,2-ef][3]benzazepine-2-carboxaldehyde**

A solution of 7-chloro-3,4,5,6-tetrahydro-4-methylthieno[4,3,2-ef][3]benzazepine-2-methanol (1.6 g,

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6 mmol) in dichloromethane (150 ml) was stirred under argon with activated manganese dioxide (8.3 g) for 2 hours. The mixture was filtered through Celite™ and the filtrate was dried with magnesium sulfate and concentrated to give a 63% yield of 7-chloro-3,4,5,6-tetrahydro-4-methylthieno[4,3,2-ef][3]benzazepine-2-carboxaldehyde.

7-Chloro-2-ethenyl-3,4,5,6-tetrahydro-4-methylthieno[4,3,2-ef][3]benzazepine (SKF-104856)
Sodium hydride (60 % dispersion in mineral oil. 3.8 mmol) was added to a stirred solution of methyltriphenylphosphonium bromide (1.35 g, 3.8 mmol) in dry tetrahydrofuran (30 ml) and stirred for 15 minutes. The mixture was treated with a solution of 7-chloro-3,4,5,6-tetrahydro-4-methylthieno[4,3,2-ef][3]benzazepine-2-carboxaldehyde, prepared as in Example 3, (0.5 g, 1.9 mmol) in dimethylformamide (4 ml), stirred at 25°C for 16 hours, quenched with ice and extracted with ethyl acetate. The organic phase was washed, dried and concentrated and the residue was chromatographed on silica gel eluted with a gradient of methylene chloride to methanol-methylene chloride (3.5:96.5). The product was treated with hydrogen chloride to give 0.2 g (35%) of 7-chloro-2-ethenyl-3,4,5,6-tetrahydro-4-methylthieno[4,3,2-ef][3]benzazepine hydrochloride: mp 234-236°C.

The following is an example of the use of the cloned Human α_1 adrenergic receptors to identify the relevant α_1 -Receptor subtype for the therapy of Benign Prostatic Hypertrophy.

Example 6

Protocol for the Determination of the Potency of α_1 Antagonists

The activity of compounds at the different human

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receptors was determined in vitro using cultured cell lines that selectively express the receptor of interest. These cell lines were prepared by transfecting the cloned cDNA or cloned genomic DNA or constructs containing both genomic DNA and cDNA encoding the human α -adrenergic, serotonin, histamine, and dopamine receptors as follows:

α_{1A} Human Adrenergic Receptor: The entire coding region of α_{1A} (1719 bp), including 150 basepairs of 5' untranslated sequence (5' UT) and 300 bp of 3' untranslated sequence (3' UT), was cloned into the BamHI and ClaI sites of the polylinker-modified eukaryotic expression vector pCEXV-3, called EXJ.HR. The construct involved the ligation of partial overlapping human lymphocyte genomic and hippocampal cDNA clones: 5' sequence were contained on a 1.2 kb SmaI-XhoI genomic fragment (the vector-derived BamHI site was used for subcloning instead of the internal insert-derived SmaI site) and 3' sequences were contained on an 1.3 kb XhoI-ClaI cDNA fragment (the ClaI site was from the vector polylinker). Stable cell lines were obtained by cotransfection with the plasmid α_{1A} /EXJ (expression vector containing the α_{1A} receptor gene) and the plasmid pGCCos3neo (plasmid containing the aminoglycoside transferase gene) into LM(tk⁻), CHO, and NIH3T3 cells, using calcium phosphate technique. The cells were grown, in a controlled environment (37°C., 5% CO₂), as monolayers in Dulbecco's modified Eagle's Medium (GIBCO, Grand Island, NY) containing 25mM glucose and supplemented with 10% bovine calf serum, 100 units/ml penicillin g, and 100 μ g/ml streptomycin sulfate. Stable clones were then selected for resistance to the antibiotic G-418 (1 mg/ml), and membranes were harvested and assayed for their ability to bind [³H]prazosin as described below (see "Radioligand Binding assays").

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α_{1B} Human Adrenergic Receptor: The entire coding region of α_{1B} (1563 bp), including 200 basepairs and 5' untranslated sequence (5' UT) and 600 bp of 3' untranslated sequence (3' UT), was cloned into the EcoRI site of pCEXV-3 eukaryotic expression vector. The construct involved ligating the full-length containing EcoRI brainstem cDNA fragment from λ ZapII into the expression vector. Stable cell lines were selected as described above.

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α_{1C} Human Adrenergic Receptor: The entire coding region of α_{1C} (1401 bp), including 400 basepairs of 5' untranslated sequence (5' UT) and 200 bp of 3' untranslated sequence (3' UT), was cloned into the KpnI site of the polylinker-modified pCEXV-3-derived eukaryotic expression vector, EXJ.RH. The construct involved ligating three partial overlapping fragments: a 5' 0.6kb HincII genomic clone, a central 1.8 EcoRI hippocampal cDNA clone, and a 3' 0.6Kb PstI genomic clone. The hippocampal cDNA fragment overlaps with the 5' and 3' genomic clones so that the HincII and PstI sites at the 5' and 3' ends of the cDNA clone, respectively, were utilized for ligation. This full-length clone was cloned into the KpnI site of the expression vector, using the 5' and 3' KpnI sites of the fragment, derived from vector (i.e., pBluescript) and 3'-untranslated sequences, respectively. Stable cell lines were selected as described above.

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Radioligand Binding Assays: Transfected cells from culture flasks were scraped into 5ml of 5mM Tris-HCl, 5mM EDTA, pH 7.5, and lysed by sonication. The cell lysates were centrifuged at 1000 rpm for 5 min at 4°C, and the supernatant was centrifuged at 30,000 x g for 20 min at 4°C. The pellet was suspended in 50mM Tris-HCl, 1mM MgCl₂, and 0.1% ascorbic acid at pH 7.5. Binding of the α_1 antagonist [³H]prazosin (0.5 nM,

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specific activity 76.2 Ci/mmol) to membrane preparations of LM(tk-) cells was done in a final volume of 0.25 ml and incubated at 37°C for 20 min. Nonspecific binding was determined in the presence of 10 μ M phentolamine. The reaction was stopped by filtration through GF/B filters using a cell harvester. Inhibition experiments, routinely consisting of 7 concentrations of the tested compounds, were analyzed using a non-linear regression curve-fitting computer program to obtain K_i values.

Example 7

Functional Properties of α_1 Antagonists in the Human Prostate

The efficacy of α_1 adrenergic antagonists for the treatment of benign prostatic hyperplasia (BPH) is related to their ability to elicit relaxation of prostate smooth muscle. An index of this efficacy can be obtained by determining the potency of α_1 antagonists to antagonize the contraction of human prostatic tissue induced by an α_1 agonist "in vitro". Furthermore, by comparing the potency of subtype selective α_1 antagonists in binding assays using human α_1 receptors with their potency to inhibit agonist-induced smooth muscle contraction, it is possible to determine which of the α_1 adrenergic receptor subtypes is involved in the contraction of prostate smooth muscle.

Methods: Prostatic adenomas were obtained at the time of surgery from patients with symptomatic BPH. These were cut into longitudinal strips of 15mm long and 2-4 mm wide, and suspended in 5ml organ baths containing Krebs buffer (pH 7.4). The baths were maintained at 37°C and continuously oxygenated with 5% CO₂ and 95% O₂. Isometric tension was measured with a Grass Instrument FT03 force transducer interfaced with a computer.

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Tissue strips were contracted with varying concentrations of phenylephrine after incubating for 20 minutes in the absence and presence of at least three different concentrations of antagonist. Dose-response curves for phenylephrine were constructed, and the antagonist potency (pA_2) was estimated by the dose-ratio method. The concentration of some antagonists in the tissue bath was assessed by measuring the displacement of [3H]prazosin by aliquots of the bath medium, using membrane preparations of the cloned human α_{1c} receptor. This control was necessary to account for losses of antagonist due to adsorption to the tissue bath and/or metabolism during the time the antagonists were equilibrated with the prostate tissue.

Results:

Table 3 shows that the pA_2 values measured for a series of α_1 antagonists in human prostate tissue correlate closely ($r=0.76$) with the corresponding pK_i values measured in the α_{1c} receptor assays. In contrast, the human prostate pA_2 values correlate poorly with the pK_i values measured at the α_{1A} ($r=-0.06$) and α_{1B} ($r=-0.24$) adrenergic receptors. (See Figure 7.) Thus, antagonists which are more potent at blocking the α_{1c} adrenergic receptor are more effective at blocking the contraction of the human prostate than antagonists which are more potent at the α_{1A} or α_{1B} adrenergic receptors. In addition, antagonists which are selective for the α_{1c} receptor will have a better therapeutic ratio than nonselective α antagonists.

Table 3.
COMPARISON OF THE BINDING POTENCY (PK_1) OF ALPHA-1 ANTAGONISTS IN CLONED HUMAN RECEPTORS
AND THEIR PROTENCY (PA_1) TO INHIBIT PROSTATE SMOOTH MUSCLE CONTRACTION

Compound	Human Alpha-1 Adrenergic (PK_1)			Human Prostate (PA_1)
	$\alpha 1A$	$\alpha 1B$	$\alpha 1C$	
1 Prazosin	9.48	9.26	9.23	9.08
3 A-30360	7.49	7.86	8.52	8.72
4 5-Methyl-Urapidil	7.79	6.77	8.35	8.38
5 Indoramin	6.74	7.39	8.35	7.86
6 SKF-104856	8.48	7.50	7.60	7.66
7 Compound 7	6.82	7.18	8.42	7.63
9 Compound 9	6.12	6.76	7.83	7.41
10 Terazosin	8.46	8.71	8.16	7.30

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References

- 1.- Borden, L.A., Maxfield, F.R., Goldman, J.E., and
Shelanski, M.L., Neurobiol. Aging., 13, 33-38,
1991.
- 5 2.- Bruno, J. F., J. Whittaker, J. Song, and M.
Berelowitz. Biochem. Biophys. Res. Comm. 179,
1485-1490 (1991).
- 10 3.- Bush, A.W., Borden, L.A., Greene, L.A., and
Maxfield, F.R., J. Neurochem. 57, 562-574, 1991.
- 15 4.- Cotecchia, S., Schwinn, D.A., Randall, R.R.,
Lefkowitz, R.J., Caron, M.G., and Kobilka, B.K.,
Proc. Natl. Acad. Sci. USA, 85, 7159-7163, 1988.
- 5.- Feinberg, A. P., and B. Vogelstein. Anal.
Biochem. 132, 6-13 (1983).
- 20 6.- Flavahan, N.A. and Vanhoutte, P.M., In: The Alpha-
1 Adrenergic Receptors, (ed. by R.R. Ruffolo, Jr.,
Humana Press, Clifton NJ) pp. 351-403, 1987.
- 25 7.- Forray, C., and El-Fakahany, E.E., Mol.
Pharmacol., 37, 893-902, 1990.
- 8.- Graziadei, I., Zernig, G., Boer, R., and Glossman,
H., Eur. J. Pharmacol. 172, 329-337, 1989.
- 30 9.- Gross, G., Hanft, G., and Rugevics, C., Eur. J.
Pharmacol., 151, 333-335, 1989.
- 35 10.- Hieble, J.P., Sarau, H.M., Foley, J.J., DeMarinis,
R.M., and Pendleton, P.G., Naunyn-Schmiedeberg's
Arch. Pharmacol., 318, 267-273, 1982.

-88-

- 11.- Langer, S.Z., *Pharmacol. Rev.*, 32, 377-360, 1980.
- 12.- Lomasney, J.W., Cotecchia, S., Lorenz, W., Leung, W.-Y., Schwinn, D.A., Yang-Feng, T.L., Brownstein, M., Lefkowitz, R.J., and Caron, M., J. Biol. Chem., 266, 6365-6369, 1991.
- 13.- Miller, J. and R. N. Germain. J. Exp. Med. 164, 1478-1489 (1986).
- 14.- Minneman, K.P., *Pharmacol. Rev.*, 40, 87-119, 1988.
- 15.- Morrow, A.L., and Creese, I., *Mol. Pharmacol.*, 29, 321-330, 1986.
- 16.- Perez, D. M., M. T. Piascik, and R. M. Graham. *Mol. Pharmacol.* 40, 876-883 (1991).
- 17.- Sambrook, J., Fritsch, E.F., and Maniatis, T., In: *Molecular Cloning: A Laboratory Manual*, 2nd Edition (Cold Spring Harbor Lab., Cold Spring Harbor, N.Y.), 1989.
- 18.- Sanger, S. *Proc. Natl. Acad. Sci. USA* 74, 5463-5467 (1977).
- 19.- Sawutz, D. G., S. M. Lanier, C. D. Warren, and R. M. Graham. *Mol. Pharmacol.* 32, 565-571 (1987).
- 20.- Schwinn, D.A., Lomasney, J.W., Lorenz, W., Szklut, P.J., Freneau, R.T., Yang-Feng, T.L., Caron, M.G., Lefkowitz, R.J. and Cotecchia, S., *J. Biol. Chem.*, 265, 8183-8189, 1990.
- 21.- Schwinn, D.A., Page, S.A., Middleton, J.P., Lorenz, W., Liggett, S.B., Yamamoto, E., Lapetina, E.G., Caron, M.G., Lefkowitz, R.J., and Cotecchia,

-89-

S., Mol. Pharmacol., 40, 619-626, 1991.

- 22.- Southern, E. M. J. Mol. Biol. 98,503-505
(1975).Starke, S., Rev. Physiol. Biochem.
5 Pharmacol., 88, 199-236, 1981.
- 23.- Timmermans, P.B.M.W.M., Karamat Ali, F., Kwa,
H.Y.,Schoop, A.M.C., Slothorst-Grisdijk, F.P., and
van Zwieten, P.A., Mol. Pharmacol., 20, 295-301,
1981.
- 10
- 24.- Timmermans, P.B.M.W.M., and Thoolen. M.J.M.C., In:
The Alpha-1 Adrenergic Receptors, (ed. by R.R.
Ruffolo, Jr., Humana Press, Clifton NJ) pp. 113-
187, 1987.
- 15
- 25.- Voigt, M. M., J. Kispert, and H. Chin. Nucleic
Acid Res. 18, 1053 (1990).
- 26.- Weinshank, R.L., Zgombick, J.M., Macchi, M.,
20 Adham, N., Lichtblau, H., Branchek, T.A., and
Hartig, P.R., Mol. OPharmacol., 38, 681-688, 1990.
- 27.- Cohen, J., (1993) J. Clin. Pharmacol., 33, 272-
278.
- 25
- 28.- Manning, A.S. and Hearse, D.J., (1984) J. Mol.
Cell Cardiol., 16: 497-518.
- 29.- Benfey, B.G., (1992) Can. J. Physiol. Pharmacol.,
30 71: 103-111.
- 30.- Cubeddu, L. X., (1984) American Heart Journal,
116: 133-161.
- 35 31.- Nishimura, K., Kuwayama, Y., Matsugi, T., Sun, N.,
and Shirasawa, E., (1993) Investigative Ophthal.
& Visual Sci., 34: 1761-1765.

-90-

- 32.- Kincaid-Smith, P., (1989) **Journal of Human Hypertension**, 2743: 75-83.
- 5 33.- Ames, R. P. and Kiyasu, J.Y., (1989) **J. Clin. Pharmacol.**, 29: 123-127.
- 34.- Pool, J.L., (1991) **Am. Heart J.**, 121: 251-260.
- 10 35.- Christ, G.J., Schwartz, C.B., Stone, B.A., Parker, M., Janis, M., Gondre, M., Valcic, M., and Melman, A., (1992) **Amer. Physiological Soc.**, H15-H20.
- 15 36.- Rosenthal, J., (1989) **Journal of Human Hypertension**, 3: 85-91.
- 37.- Kowala, M.C. and Nicolosi, R.J., (1989) **Journal of Cardiovascular Pharm.**, 13: 545-549.
- 20 38.- Nash, D.T., (1990) **Clin. Cardiol.**, 13: 764-772.
- 39.- Waite, M.A., (1991) **Journal of Internal Medicine**, 229: 113-117.
- 25 40.- Achari, R. and Laddu, A., (1992) **J. Clin. Pharm.**, 32: 520-523.
- 41.- Kowala, M.C., Nunnari, J.J., Durham, S.K., and Nicolosi, R.J., (1991) **Atherosclerosis**, 91: 35-39.
- 30 42.- Krupp, M.N., Hoover, K.W., and Valentine, J.J., (1989) **Journal of Cardiovascular Pharm.**, 13: 511-519.
- 35 43.- Jansen, H., Lammers, R., Baggen, M.G.A., and Birkenhäger, J.C., (1989) **Journal of Cardiovascular Pharm.**, 13: S5-S10.

-91-

- 44.- Joint National Committee on Detection, Evaluation,
and Treatment of High Blood Pressure, (1993)
Archives of Internal Medicine, 163: 164-183.
- 5 45.- Sourander, L.B., (1990) **Gerontology**, 36: 19-25.
- 46.- Empey, D.W. and Medder, K.T., (1988) **Drugs**, 35:
438-443.
- 10 47.- Andersson, K.E., (1988) **Drugs**, 35: 477-494.
- 48.- MacDonald, E., Ruskoaho, H., Scheinen, M. and
Virtanen, R., (1988) **Annals of Clin. Res.**, 20:
298-310.
- 15 49.- Lepor, H., R. Tan, S. Meretyk, and E. Shapiro.
Alpha₁ adrenoceptor subtypes in the human
prostate. **J. Urol.** 149:640-642 (1993).
- 50.- Lepor, H., S. Auerbach, A Puras-Baez, P. Narayan,
20 M. Soloway, F. Lowe, T. Moon, G. Leifer, and P.
Madsen. A randomized, placebo-controlled
multicenter study of the efficacy and safety of
teazosin in the treatment of benign porstatic
hyperplasia. **J. Urol.** 148:1467-4174 (1992).
- 25 51.- Price, D.T., R.J. Lefkowitz, M.G. Caron, and D.A.
Schwinn. Alpha₁-adrenergic receptor mRNA
expression in human tissues. **FASEB J.** 7:A141
(1993).

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What is claimed:

1. An isolated nucleic acid molecule encoding a human α_1 adrenergic receptor.
- 5 2. A nucleic acid molecule of claim 1, wherein the nucleic acid molecule encodes a human α_{1a} adrenergic receptor.
- 10 3. A nucleic acid molecule of claim 1, wherein the nucleic acid molecule encodes a human α_{1b} adrenergic receptor.
- 15 4. A nucleic acid molecule of claim 1, wherein the nucleic acid encodes a human α_{1c} adrenergic receptor.
- 20 5. A nucleic acid molecule of claim 1, wherein the nucleic acid molecule is a DNA molecule.
- 25 6. A DNA molecule of claim 5, wherein the DNA molecule is a cDNA molecule.
7. A nucleic acid molecule of claim 1, wherein the nucleic acid molecule has been so mutated that the human α_1 adrenergic receptor encoded by the nucleic acid molecule is incapable of receptor activity.
- 30 8. A nucleic acid molecule of claim 7, wherein the nucleic acid molecule is a DNA molecule.
9. A DNA molecule of claim 8, wherein the DNA molecule is a cDNA molecule.
- 35 10. A vector comprising a DNA molecule of claim 5.

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11. A plasmid comprising the vector of claim 10.
12. A vector of claim 10 adapted for expression in a bacterial cell which comprises the regulatory elements necessary for expression of the DNA in a bacterial cell so located relative to the DNA encoding a human α_1 adrenergic receptor as to permit expression thereof.
13. A vector of claim 10 adapted for expression in a yeast cell which comprises the regulatory elements necessary for the expression of the DNA in a yeast cell so located relative to the DNA encoding a human α_1 adrenergic receptor as to permit expression thereof.
14. A vector of claim 10 adapted for expression in a mammalian cell which comprises the regulatory elements necessary for expression of the DNA in the mammalian cell so located relative to the DNA encoding a human α_1 adrenergic receptor as to permit expression thereof.
15. A plasmid of claim 11 adapted for expression in a mammalian cell which comprises the regulatory elements necessary for expression of the DNA in the mammalian cell so located relative to the DNA encoding a human α_1 adrenergic receptor as to permit expression thereof.
16. A plasmid designated pCEXV- α_{1a} .
17. A plasmid designated pcEXV- α_{1b} .
18. A plasmid designated pcEXV- α_{1c} .

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19. A mammalian cell comprising the plasmid of claim 11.
- 5 20. A mammalian cell of claim 19, wherein the mammalian cell is an LM (tk-) cell.
21. An LM (tk-) cell comprising the plasmid of claim 15.
- 10 22. A nucleic acid probe comprising a nucleic acid molecule of at least 15 nucleotides capable of specifically hybridizing with a sequence included within the sequence of a nucleic acid molecule encoding a human α_{1a} receptor.
- 15 23. A nucleic acid probe comprising a nucleic acid molecule of at least 15 nucleotides capable of specifically hybridizing with a sequence included within the sequence of a nucleic acid molecule encoding a human α_{1b} receptor.
- 20 24. A nucleic acid probe comprising a nucleic acid molecule of at least 15 nucleotides capable of specifically hybridizing with a sequence included within the sequence of a nucleic acid molecule encoding a human α_{1c} receptor.
- 25 25. The nucleic acid probe of claim 22, 23, or 24, wherein the nucleic acid is DNA.
- 30 26. A nucleic acid probe of claim 25, which comprises degenerate oligonucleotides.
- 35 27. An antisense oligonucleotide having a sequence capable of specifically binding to a mRNA molecule encoding a human α_{1a} adrenergic receptor so as to prevent translation of the mRNA molecule.

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28. An antisense oligonucleotide having a sequence capable of specifically binding to a mRNA molecule encoding a human α_{1b} adrenergic receptor so as to prevent translation of the mRNA molecule.
- 5
29. An antisense oligonucleotide having a sequence capable of specifically binding to a mRNA molecule encoding a human α_{1c} adrenergic receptor so as to prevent translation of the mRNA molecule.
- 10
30. An antisense oligonucleotide having a sequence capable of binding specifically to a cDNA molecule of claim 6.
- 15
31. Antisense oligonucleotides comprising degenerate oligonucleotides of an antisense oligonucleotide of claims 27, 28, or 29.
- 20
32. An antisense oligonucleotides of claims 27, 28, or 29 comprising chemical analogs of nucleotides.
- 25
33. A method for detecting expression of a specific human α_1 adrenergic receptor, which comprises obtaining RNA from cells or tissue, contacting the RNA so obtained with a nucleic acid probe of claim 22, 23 or 24 under hybridizing conditions, detecting the presence of any mRNA hybridized to the probe, the presence of mRNA hybridized to the probe indicating expression of the specific human α_1 adrenergic receptor, and thereby detecting the expression of the specific human α_1 adrenergic receptor.
- 30
34. A method of detecting expression of a specific human α_1 adrenergic receptor in a cell or tissue by in situ hybridization, contacting the cell or tissue with a nucleic acid probe of claim 25 or an
- 35

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- antisense oligonucleotide of claims 27, 28 or 29 under hybridizing conditions, detecting the presence of any mRNA hybridized to the probe, the presence of mRNA hybridized to the probe indicating expression of the specific human α_1 adrenergic receptor, and thereby detecting the expression of the specific human α_1 adrenergic receptor.
- 5
- 10 35. A method of isolating a gene encoding a receptor by nucleic acid sequence homology using a nucleic acid probe of claims 25 or 26.
- 15 36. A method of claim 35, which comprises using the the polymerase chain reaction to obtain a DNA molecule by nucleic acid sequence homology, the DNA molecule of which is used to isolate a gene encoding a receptor.
- 20 37. A nucleic acid molecule comprising the gene identified by the method of claims 35 or 36.
- 25 38. A method of isolating DNA of claim 5, which comprises growing bacteria transformed with a plasmid comprising the DNA of claim 5, lysing the cells and purifying the DNA from the lysed cells.
- 30 39. A nucleic acid molecule of claim 1, wherein the nucleic acid has been so mutated within a 5' transcriptional regulatory element or other stability, processing, transcription, or translation-determining region within the 5' or 3' untranslated region of the DNA so as to increase the stability of the mRNA or to enhance the processing, transcription, or translation of the RNA.
- 35

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40. A nucleic acid molecule of claim 1, wherein the nucleic acid has been so mutated within a 5' transcriptional regulatory element or other stability, processing, transcription, or translation-determining region within the 5' or 3'untranslated region of the DNA so as to decrease the stability of the mRNA or to diminish the processing, transcription, or translation of the RNA.
41. An isolated human α_1 adrenergic receptor protein.
42. An isolated human α_1 adrenergic receptor protein of claim 41, wherein the human α_1 adrenergic receptor protein is the human α_{1a} adrenergic receptor protein.
43. An isolated human α_1 adrenergic receptor protein of claim 41, wherein the human α_1 adrenergic receptor protein is the human α_{1b} adrenergic receptor protein.
44. An isolated human α_1 adrenergic receptor protein of claim 41, wherein the human α_1 adrenergic receptor protein is the human α_{1c} adrenergic receptor protein.
45. A method of preparing a human α_1 adrenergic receptor protein of claim 41, which comprises inducing cells to express the human α_1 adrenergic receptor protein, recovering the human α_1 adrenergic receptor from the resulting cells, and purifying the human α_1 adrenergic receptor so recovered.
46. A method of preparing a human α_1 adrenergic receptor of claim 41, which comprises inserting a

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- 5 nucleic acid molecule encoding the human α_1 adrenergic receptor in a suitable vector, inserting the resulting vector in suitable host cell, recovering the human α_1 adrenergic receptor produced by the resulting cell, and purifying the human α_1 adrenergic receptor so recovered.
- 10 47. An antibody directed to a human α_{1a} adrenergic receptor or to a protein fragment of the human α_{1a} adrenergic receptor.
- 15 48. An antibody directed to a human α_{1b} adrenergic receptor or to a protein fragment of the human α_{1b} adrenergic receptor.
49. An antibody directed to a human α_{1c} adrenergic receptor or a protein fragment of the human α_{1c} adrenergic receptor.
- 20 50. An antibody of claims 47, 48 or 49 wherein the antibody is a monoclonal antibody.
- 25 51. A monoclonal antibody of claim 50 wherein the antibody is directed to an epitope of a human cell-surface α_1 adrenergic receptor and having an amino acid sequence substantially the same as the amino acid sequence for a cell-surface epitope of the human α_1 adrenergic receptor.
- 30 52. A pharmaceutical composition comprising an amount of a substance effective to alleviate the abnormalities resulting from overexpression of a human α_{1a} adrenergic receptor and a pharmaceutically acceptable carrier.
- 35 53. A pharmaceutical composition comprising an amount of a substance effective to alleviate the

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abnormalities resulting from overexpression of a human α_{1b} adrenergic receptor and a pharmaceutically acceptable carrier.

- 5 54. A pharmaceutical composition comprising an amount of a substance effective to alleviate the abnormalities resulting from overexpression of a human α_{1c} adrenergic receptor and a pharmaceutically acceptable carrier.
- 10
- 15 55. A pharmaceutical composition comprising an amount of a substance effective to alleviate abnormalities resulting from underexpression of a human α_{1a} adrenergic receptor and a pharmaceutically acceptable carrier.
- 20 56. A pharmaceutical composition comprising an amount of a substance effective to alleviate abnormalities resulting from underexpression of a human α_{1b} adrenergic receptor and a pharmaceutically acceptable carrier.
- 25 57. A pharmaceutical composition comprising an amount of a substance effective to alleviate abnormalities resulting from underexpression of a human α_{1c} adrenergic receptor and a pharmaceutically acceptable carrier.
- 30 58. A pharmaceutical composition comprising an effective amount of an oligonucleotide of claim 27 effective to reduce expression of a human α_{1a} adrenergic receptor by passing through a cell membrane and specifically binding with mRNA encoding a human α_{1a} adrenergic receptor in the cell so as to prevent its translation and a pharmaceutically acceptable hydrophobic carrier capable of passing through a cell membrane.
- 35

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59. A pharmaceutical composition comprising an effective amount of an oligonucleotide of claim 28 effective to reduce expression of a human α_{1b} adrenergic receptor by passing through a cell membrane and specifically binding with mRNA encoding a human α_{1b} adrenergic receptor in the cell so as to prevent its translation and a pharmaceutically acceptable hydrophobic carrier.
60. A pharmaceutical composition comprising an effective amount of an oligonucleotide of claim 29 effective to reduce expression of a human α_{1c} adrenergic receptor by passing through a cell membrane and specifically binding with mRNA encoding a human α_{1c} adrenergic receptor in the cell so as to prevent its translation and a pharmaceutically acceptable hydrophobic carrier.
61. A pharmaceutical composition claims 58, 59 or 60, wherein the nucleotide is coupled to a substance which inactivates mRNA.
62. A pharmaceutical composition of claim 61, wherein the substance which inactivates the mRNA is a ribozyme.
63. A pharmaceutical composition of claim 61, wherein the pharmaceutically acceptable hydrophobic carrier capable of passing through a cell membrane comprises a structure which binds to a transporter specific for a selected cell type and is thereby taken up by the cells of the selected cell type.
64. A pharmaceutical composition which comprises an amount of the antibody of claim 47 effective to block binding of naturally occurring substrates to a human α_{1a} adrenergic receptor and a

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pharmaceutically acceptable carrier.

- 5 65. A pharmaceutical composition which comprises an amount of the antibody of claim 48 effective to block binding of naturally occurring substrates to a human α_{1b} adrenergic receptor and a pharmaceutically acceptable carrier.
- 10 66. A pharmaceutical composition which comprises an amount of the antibody of claim 49 effective to block binding of naturally occurring substrates to a human α_{1c} adrenergic receptor and a pharmaceutically acceptable carrier.
- 15 67. A transgenic nonhuman mammal which comprises a nucleic acid molecule of claim 1.
- 20 68. A transgenic nonhuman mammal which comprises the DNA molecule of claim 39.
- 25 69. A transgenic nonhuman mammal which comprises the nucleic acid molecule of claim 7.
- 30 70. A transgenic nonhuman mammal which comprises the DNA molecule of claim 40.
- 35 71. A transgenic nonhuman mammal whose genome comprises a nucleic acid molecule of claim 1 so placed as to be transcribed into antisense mRNA complementary to mRNA encoding a human α_1 adrenergic receptor and which hybridizes to mRNA encoding a human α_1 adrenergic receptor thereby reducing its translation.
72. The transgenic nonhuman mammal of any of claims 67, 68, 69, 70, or 71, wherein the nucleic acid molecule further comprises an inducible promoter.

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73. The transgenic nonhuman mammal of any of claims 67, 68, 69, 70, 71, or 72 wherein the nucleic molecule additionally comprises tissue specific regulatory elements.
- 5
74. The transgenic non-human mammal of any of claims 67, 68, 69, 70, 71, 72, or 73, wherein the transgenic non-human mammal is a mouse.
- 10
75. A method of determining the physiological effects of varying the levels of expression of a specific human α_1 adrenergic receptor which comprises producing a transgenic non-human mammal whose levels of expression of a human α_1 adrenergic receptor can be varied by use of an inducible promoter.
- 15
76. A method of determining the physiological effects of expressing varying levels of a specific human α_1 adrenergic receptor which comprises producing a panel of transgenic non-human mammals each expressing a different amount of a human α_1 adrenergic receptor.
- 20
77. A method of determining whether a ligand not known to be capable of specifically binding to a human α_1 adrenergic receptor can specifically bind to a human α_1 adrenergic receptor, which comprises contacting a mammalian cell comprising a plasmid which further comprises a DNA molecule adapted for expression in a mammalian cell which allows subject cell to express a human α_1 adrenergic receptor on the cell surface with the ligand under conditions permitting binding of ligands known to bind to a human α_1 adrenergic receptor, detecting the presence of any ligand bound to the human α_1 adrenergic receptor, the presence of bound ligand
- 25
- 30
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thereby determining the ligand binds to the human α_1 adrenergic receptor, and thereby determining whether the ligand binds to the human α_1 adrenergic receptor.

5

78. The method of claim 77, wherein the receptor is a human α_{1a} adrenergic receptor.

10

79. The method of claim 77, wherein the receptor is a human α_{1b} adrenergic receptor.

80. The method of claim 77, wherein the receptor is a human α_{1c} adrenergic receptor.

15

81. The method of claims 78, 79 or 80 wherein the mammalian cell is a non-neuronal cell.

20

82. A method of screening drugs to identify drugs which interact with, and bind to, a human α_1 adrenergic receptor on the surface of a cell, which comprises contacting a mammalian cell which comprises a plasmid adapted for expression in a mammalian cell which further comprises a DNA molecule which expresses a human α_1 adrenergic receptor on the cell surface with a plurality of drugs, determining those drugs which bind to the human α_{1a} adrenergic receptor expressed on the cell surface of the mammalian cell, and thereby identifying drugs which interact with, and bind to, the human α_1 adrenergic receptor.

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83. The method of claim 82, wherein the receptor is a human α_{1a} adrenergic receptor.

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84. The method of claim 82, wherein the receptor is a human α_{1b} adrenergic receptor.

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85. The method of claim 82, wherein the receptor is a human α_{1c} adrenergic receptor.
- 5 86. The method of claims 83, 84 or 85, wherein the mammalian cell is a non-neuronal cell.
- 10 87. A method of determining whether a ligand not known to be capable of binding to a human α_1 adrenergic receptor can bind to a human α_1 adrenergic receptor, which comprises preparing a cell extract from mammalian cells, which comprise a plasmid adapted for expression in a mammal, which further comprise a DNA molecule which expresses a human α_1 adrenergic receptor on the cell surface, isolating
15 a membrane fraction from the cell extract, incubating the ligand with the membrane fraction under conditions permitting binding of ligands known to bind to the human α_1 adrenergic receptor, detecting the presence of any bound ligand, and
20 thereby determining whether the ligand binds to the human α_1 adrenergic receptor.
- 25 88. The method of claim 87, wherein the human α_1 adrenergic receptor is a human α_{1a} adrenergic receptor.
- 30 89. The method of claim 87, wherein the human α_1 adrenergic receptor is a human α_{1b} adrenergic receptor.
- 35 90. The method of claim 87, wherein the human α_1 adrenergic receptor is a human α_{1c} adrenergic receptor.
91. The method of claims 88, 89 or 90, wherein the mammalian cell is a non-neuronal cell.

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- 5 92. A method of screening drugs to identify drugs that interact with, and bind to, an α_1 adrenergic receptor, which comprises preparing a cell extract from mammalian cells, which comprise a plasmid adapted for expression in a mammalian cell which further comprise a DNA molecule which expresses a human α_1 adrenergic receptor on the cell surface, isolating a membrane fraction from the cell extract, incubating the membrane fraction with a plurality of drugs, determining those drugs which interact with and bind to the human α_1 adrenergic receptor, and thereby identifying drugs which interact with, and bind to, the human α_1 adrenergic receptor.
- 10 93. The method claim 92, wherein the receptor is a human α_{1a} adrenergic receptor.
- 15 94. The method of claim 92, wherein the receptor is a human α_{1b} adrenergic receptor.
- 20 95. The method of claim 92, wherein the receptor is a human α_{1c} adrenergic receptor.
- 25 96. The method of claims 93, 94, or 95, wherein the mammalian cell is a non-neuronal cell.
- 30 97. A method of identifying a ligand which interacts with, and activates or blocks the activation of, a human α_1 adrenergic receptor on the surface of a cell, which comprises contacting a mammalian cell which comprises a plasmid adapted for expression in a mammalian cell which further comprises a DNA molecule which expresses a human α_1 adrenergic receptor on the cell surface with the ligand, determining whether the ligand activates or blocks the activation of the receptor
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using a bioassay such as second messenger assays, and thereby identifying a ligand which interacts with, and activates or blocks the activation of, a human α_1 adrenergic receptor.

5

98. The method of claim 97, wherein the human α_1 adrenergic receptor is a human α_{1a} adrenergic receptor.

10

99. The method of claim 97, wherein the human α_1 adrenergic receptor is a human α_{1b} adrenergic receptor.

15

100. The method of claim 97, wherein the human α_1 adrenergic receptor is a human α_{1c} adrenergic receptor.

20

101. The method of claims 98, 99 or 100, wherein the cell is a non-neuronal cell comprising the cellular components required to produce the second messenger which is being identified.

25

102. The method of claim 97, wherein the ligand is a drug.

30

103. A method for identifying a ligand which is capable of binding to and activating or inhibiting a human α_1 adrenergic receptor, which comprises contacting a mammalian cell, wherein the membrane lipids have been labelled by prior incubation with a labelled myo-inositol phosphate molecule, the mammalian cell comprising a plasmid adapted for expression in a mammalian cell which further comprises a DNA molecule which expresses a human α_1 adrenergic receptor with the ligand and identifying an inositol phosphate metabolite released from the membrane lipid as a result of ligand binding to

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and activating an α_1 adrenergic receptor.

- 5 104. The method of claim 103, wherein the human α_1 adrenergic receptor is a human α_{1a} adrenergic receptor.
- 10 105. The method of claim 103, wherein the human α_1 adrenergic receptor is a human α_{1b} adrenergic receptor.
- 15 106. The method of claim 103, wherein the human α_1 adrenergic receptor is a human α_{1c} adrenergic receptor.
- 20 107. The method of claims 104, 105, or 106, wherein the cell is a non-neuronal cell expressing the cellular components required to produce the second messenger which is being identified.
- 25 108. The method of claim 103 wherein the ligand is a drug.
- 30 109. A method for identifying a ligand that is capable of binding to and activating or inhibiting a human α_1 adrenergic receptor, wherein the binding of ligand to the adrenergic receptor results in a physiological response, which comprises contacting a mammalian cell which comprises a plasmid adapted for expression in a mammalian cell which further comprises a DNA molecule which expresses a human α_1 adrenergic receptor with a calcium sensitive fluorescent indicator, removing the indicator that has not been taken up by the cell, contacting the cells with the ligand and identifying an increase or decrease in intracellular Ca^{+2} as a result of ligand binding to and activating the receptor.
- 35

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110. The method of claim 109, wherein the human α_1 adrenergic receptor is a human α_{1a} adrenergic receptor.
- 5 111. The method of claim 109, wherein the human α_1 adrenergic receptor is a human α_{1b} adrenergic receptor.
- 10 112. The method of claim 109, wherein the human α_1 adrenergic receptor is a human α_{1c} adrenergic receptor.
- 15 113. The method of claim 110, 111 or 112, wherein the cell is a non-neuronal cell expressing the cellular components required to produce the second messenger which is being identified.
- 20 114. The method of claim 108, wherein the ligand is a drug.
- 25 115. A ligand identified by the methods of claims 77, 87, 97, 103 or 109.
- 30 116. A pharmaceutical composition of a drug identified by the methods of claims 82, 92, 102, 108 or 114.
- 35 117. A method for detecting the presence of a human α_{1a} adrenergic receptor on the surface of a cell, which comprises contacting the cell with an antibody of claim 47, under conditions that permit binding of the antibody to the receptor, detecting the presence of any of the antibody bound to the cell, and thereby the presence of a human α_{1a} adrenergic receptor on the surface of the cell.
118. A method for detecting the presence of a human α_{1b} adrenergic receptor on the surface of a cell,

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5 which comprises contacting the cell with an antibody of claim 48, under conditions that permit binding of the antibody to the receptor, detecting the presence of any of the antibody bound to the cell, and thereby the presence of a human α_{1b} adrenergic receptor on the surface of the cell.

10 119. A method for detecting the presence of a human α_{1c} adrenergic receptor on the surface of a cell, which comprises contacting the cell with an antibody of claim 49, under conditions that permit binding of the antibody to the receptor, detecting the presence of any of the antibody bound to the cell, and thereby the presence of a human α_{1c} adrenergic receptor on the surface of the cell.

20 120. A method for treating an abnormal condition related to an excess of activity of a human α_1 adrenergic receptor subtype, which comprises administering a patient an amount of a pharmaceutical composition of claim 116, effective to reduce α_1 adrenergic activity as a result of naturally occurring substrate binding to and activating a specific α_1 adrenergic receptor.

25 121. The method of claim 120, wherein the condition is benign prostatic hypertrophy.

30 122. The method of claim 120, wherein the condition is coronary heart disease.

123. The method of claim 120, wherein the condition is insulin resistance.

35 124. The method of claim 120, wherein the condition is hypertension.

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125. The method of claim 120, wherein the condition is urinary retention.
- 5 126. The method of claim 120, wherein the condition is glaucoma.
127. The method of claim 120, wherein the condition is erectile dysfunction.
- 10 128. The method of claim 120, wherein the condition is Reynaud's syndrome.
129. The method of treating abnormalities which are alleviated by an increase in the activity of a
15 specific human α_1 adrenergic receptor, which comprises administering a patient an amount of a pharmaceutical composition of claim 116, effective to increase the activity of the specific human α_1 adrenergic receptor thereby alleviating
20 abnormalities resulting from abnormally low receptor activity.
130. The method of claim 129, wherein the condition is urinary incontinence.
- 25 131. The method of claim 129, wherein the condition is nasal congestion.
132. The method of claim 129, wherein the condition is
30 hypotension.
133. A method for diagnosing a predisposition to a disorder associated with the expression of a
35 specific human α_1 adrenergic receptor allele which comprises:
- a. obtaining DNA from subjects suffering from a

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disorder;

- 5 b. performing a restriction digest of the DNA
with a panel of restriction enzymes;
- c. electrophoretically separating the resulting
DNA fragments on a sizing gel;
- 10 d. contacting the gel with a nucleic acid probe
of claim 22, 23, or 24 labelled with a
detectable marker;
- 15 e. detecting the labelled bands which have
hybridized to the DNA encoding either an α_{1a} ,
 α_{1b} or α_{1c} adrenergic receptor, labelled with
the detectable marker to create a unique
band pattern specific to the DNA of subjects
suffering with the disorder;
- 20 f. preparing DNA for diagnosis by steps a-e;
- g. comparing the unique band pattern specific
to the DNA of patients suffering from the
disorder from step e and DNA obtained for
25 diagnosis from step f to determine whether
the patterns are the same or different and
to diagnose thereby predisposition to the
disorder if the patterns are the same.
- 30 134. The method of claim 133, wherein a disorder
associated with the expression of a specific human
 α_1 adrenergic allele is diagnosed.
- 35 135. A method of identifying a substance capable of
alleviating the abnormalities resulting from
overexpression of a specific human α_1 adrenergic
receptor which comprises administering a substance

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5 to the transgenic non-human mammal of claims 67, 68, 72, or 73, and determining whether the substance alleviates the physical and behavioral abnormalities displayed by the transgenic nonhuman mammal as a result of overexpression of the human α_1 adrenergic receptor subtype.

10 136. A method of identifying a substance capable of alleviating the abnormalities resulting from underexpression of a human α_1 adrenergic receptor subtype, which comprises administering a substance to the transgenic mammal of claims 69 or 70, and determining whether the substance alleviates the physical and behavioral abnormalities displayed by the transgenic nonhuman mammal as a result of underexpression of a human α_1 adrenergic receptor subtype.

15 137. A method of treating abnormalities in a subject, wherein the abnormality is alleviated by the reduced expression of a human α_1 adrenergic receptor subtype which comprises administering to a subject an effective amount of the pharmaceutical composition of claims 52, 53, 54, 25 58, 59, 60, 64, 65, 66, 115 or 116 effective to reduce expression of the α_1 adrenergic receptor subtype.

30 138. A method of treating abnormalities resulting from underexpression of a human α_1 adrenergic receptor which comprises administering to a subject an amount of a pharmaceutical composition of claim 55, 56, 57, 115, or 116, effective to alleviate abnormalities resulting from underexpression of 35 the human α_1 adrenergic receptor.

139. The method of claim 120, wherein the condition is

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atherosclerosis.

140. The method of claim 120, wherein the condition is cardiac arrhythmias.

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141. The method of claim 120, wherein the condition is sympathetic dystrophy syndrome.

142. The method of claim 126, wherein the condition is congestive heart failure.

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FIGURE 1C

304	310	330	350	363
102	TTCTGGCAGCCTTCATCCTTATGGCCGTGGCAGGTAAACCTGCTTGTCATCCTCTCAGTG			121
	F L A A F I L M A V A G N L L V I L S V			
364	370	390	410	423
122	GCCTGCAACCGCCACCTGCAGACCGTCACCAACTATTTCATCGTGAACCTGGCCGTGGCC			141
	A C N R H L Q T V T N Y F I V N L A V A			
424	430	450	470	483
142	GACCTGCTGTGAGCGCCACCGTACTGCCCTTCTCGGCCACCATGGAGGTTCTGGGCTTC			161
	D L L L S A T V L P F S A T M E V L G F			
484	490	510	530	543
162	TGGCCCTTTGGCCCGCCCTTCTGCGACGTATGGCCCGCCGTGGACGTGTGTGCTGCACG			181
	W A F G R A F C D V W A A V D V L C C T			

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FIGURE 1D

544	GCCTCCATCCTCAGCCCTCTGCACCATCTCCGTGGACCGGTACGTGGCGGTGGCCACTCA	570	590	603
182	A S I L S L C T I S V D R Y V G V R H S			201
604	CTCAAGTACCCAGCCATCATGACCGAGCGCAAGCGCGCCCATCCTGGCCCTGCTCTGG	610	630	650
202	L K Y P A I M T E R K A A A I L A L L W			663
				221
664	GTCGTAGCCCTGGTGTCCGTAGGCCCCCTGCTGGGCTGGAAGAGCCCGTGCCCCCT	670	690	710
222	V V A L V V S V G P L L G W K E P V P			723
				241
724	GACGAGCGCTTCTGGGTATCACCGAGGCGGGCTACGCTGTCTTCTCCTCCGTGTGC	730	750	770
242	D E R F C G I T E E A G Y A V F S S V C			783
				261

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FIGURE 1E

784	790	810	830	843
262	TCCTTCTACCTGCCCATGGCGGTCA	TGTTGGTCA	TGTA	TGCGGTGACGTGGTCGCG
	S F Y L P M A V I V V M Y C R V Y V A			281
	850	870	890	
844	CGCAGCACCA	CGCGCAGCCTCGAGGCGGCGTCAAGCGCGAGGCAAGGCTCCGAG		903
282	R S T T R S L E A G V K R E R G K A S E			301
	910	930	950	
904	GTGGTGCTGGCATCCACTGTGCGGGCGGGCCACGGGCGCGGCGGCGCACGGCATG			963
302	V V L R I H C R G A A T G A D G A H G M			321
	970	990	1010	
964	CGCAGCGCCAAGGGCCACACCTTCCGCAGCTCGCTCTCCGTGCGGCTGCTCAAGTTCTCC			1023
322	R S A K G H T F R S S L S V R L L K F S			341

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FIGURE 1F

1024	CGTGAGAAAGCGGCCAAGACTCTGGCCCATCGTCTGGGTGTCTTCGTGCTCTGCTGG	1070	1083
342	R E K A A K T L A I V V G V F V L C W		361
1090	1110	1130	
1084	TTCCCTTTCTTCTTGTCTCCGCTCGGCTCCTTGTCCCGCAGCTGAAGCCATCGGAG		1143
362	F P F F V L P L G S L F P Q L K P S E		381
1150	1170	1190	
1144	GGCGTCTTCAAGGTCATCTTCTGGCTCGGCTACTTCAACAGCTGCGTGAACCCGCTCATC		1203
382	G V F K V I F W L G Y F N S C V N P L I		401
1210	1230	1250	
1204	TACCCCTGTTCAGCCGCGAGTTCAAGCGCGCCTTCCCTCCGTCTCCTGCGCTGCCAGTGC		1263
402	Y P C S S R E F K R A F L R L L R C Q C		421

FIGURE 1G

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1264	CGTCGTCGCGCGCGCGCCCTCTCTGCGGTCTACGGCCACCACTGGCGGGCCTCC	1290	1310	1323
422	R R R R R R R P L W R V Y G H H W R A S			441
1324	ACCAGCGGCCTGCGGCCAGGACTGCGCCCCCGAGTTCGGGCGGACGCGCCCCCGAGCGCCG	1330	1350	1383
442	T S G L R Q D C A P S S G D A P P G A P		1370	461
1384	CTGGCCCTCACCGCGCTCCCCGACCCCGACCCCGAACCCCGACGCCCGAGATGCAG	1390	1410	1443
462	L A L T A L P D P D P E P P G T P E M Q		1430	481
1444	GCTCCGGTCGCCAGCGCGTAAAGCCACCCAGCGCCTTCCCGGAGTGGAGGCTGCTGGGG	1450	1470	1503
482	A P V A S R R R K P P S A F R E W R L L G		1490	501

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FIGURE 1H

1504	CCGTTCCGGAGACCCACGACCCAGCTGCGGCCAAAGTCTCCAGCCCTGTCGCACAAGATC	1550	1563
502	P F R R P T T Q L R A K V S S L S H K I		521
1570		1590	1610
1564	CGCGCCGGGGCGCGCAGCGCGCAGAGGCAGCGTGCGGCCAGCGCTCAGAGGTGGAGGCT		1623
522	R A G G A Q R A E A A C A Q R S E V E A		541
1630		1650	1670
1624	GTGTCCCTAGGCGTCCACACGAGGTGGCCGAGGGCGCCACCTGCCAGGCCCTACGAATTG		1683
542	V S L G V P H E V A E G A T C Q A Y E L		561
1690		1710	1730
1684	GCCGACTACAGCAACCTACGGGAGACCGATATTTAAGGACCCAGAGCTAGGCCGCGGAG		1743
562	A D Y S N L R E T D I *		572

FIGURE 11

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1744	1750	1770	1790	1803
	TGTGCTGGGCTTGGGGGTAAGGGGAC	CAGAGAGCGGGCTGGTGT	TCTAAGAGCCCCCG	
1804	1810	1830	1850	1863
	TGCAATCGGAGACCCGAACTGATCAGGGCAGCTGCTCTGTGACATCCCTGAGGAACT			
1864	1870	1890	1910	1923
	GGCAGAGCTTGAGGCTGGAGCCCTTGAAAGGTGAAAGTAGTGGGGCCCCCTGCTGGAC			
1924	1930	1950		
	TCAGGTGCCCAGAACTCTTTTCTTAGAAGGAGAGGCTGC			

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FIGURE 2A

FIGURE 2A, 10/37
FIGURE 2B, 11/37
FIGURE 2C, 12/37
FIGURE 2D, 13/37
FIGURE 2E, 14/37
FIGURE 2F, 15/37
FIGURE 2G, 16/37
FIGURE 2H, 17/37

120	-100	-80	
-122	GCCAGGAGGGCGCCTCTGGGAAGAAGACCACGGGGGAAGCAAAGTTTCAGGGCAGCTGAG		-63
-60	-40	-20	
-62	GAGCCTTCGCCGCGAGCCCTTCCGAGGCCCAATCATCCCCAGGCTATGGAGGGCGGACTCT		-3
0	20	40	
-2	AAGATGAATCCCGACCTGGACACCGGGCCACAAACATCAGCACCTGCCCACTGGGGAGAG		57
0	M N P D L D T G H N T S A P A H W G E		19
60	80	100	
58	TTGAAATAATGCCAACTTCACTGGCCCCAACCGAGACCTCGAGCAACTCCACACTGCCCCAG		117
20	L K N A N F T G P N Q T S S N S T L P Q		39

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FIGURE 2B

120	CTGGACATCACCAGGCCATCTCTGTGGCCCTGGTGGCGCCCTTCATCCCTTTGGCC	160	177
118	L D I T R A I S V G L V L G A F I L F A		59
40			
180	ATCGTGGGCAACATCCTAGTCATCTTGTCTGTGGCCCTGCAACCGGCACCTGCGGACGCCC	220	237
178	I V G N I L V I L S V A C N R H L R T P		79
60			
240	ACCAACTACTTCAATTGTCAACCTGGCCATGGCCGACCTGCTGTTGAGCTTACCGTCCTG	280	297
238	T N Y F I V N L A M A D L L L S F T V L		99
80			
300	CCCTTCTCAGCGGCCCTAGAGGTGCTCGGCTACTGGGTGCTGGGGCGGATCTTCTGTGAC	340	357
298	P F S A A L E V L G Y W V L G R I F C D		119
100			

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FIGURE 2C

358	360	380	400	417
120	ATCTGGGCAGCCGTTGGATGTCCTGTGCTGCACAGCGTCCATTTCTGAGCCCTGTGCGCCATC			139
	I W A A V D V L C C T A S I L S L C A I			
418	420	440	460	477
140	TCCATCGATCGCTACATCGGGGTGGCTACTCTCTGCAGTATCCCACGCTGGTCAACCCGG			159
	S I D R Y I G V R Y S L Q Y P T L V T R			
478	480	500	520	537
160	AGGAAGGCCATCTTGGCGCTGCTCAGTGTCTGGGTCTTGTCCACCGTCATCTCCATCGGG			179
	R K A I L A L L S V W V L S T V I S I G			
538	540	560	580	597
180	CCTCTCCTTGGGTGGAAGGAGCGCGGCCACCCAAACGATGACAAGGAGTGCGGGGTCAACCGAA			199
	P L L G W K E P A P N D D K E C G V T E			

SUBSTITUTE SHEET

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FIGURE 2E

838	840	860	880	897
280	GCTGTCAAACCTTTTAAAGTTCTCCAGGGAAGAAAGCAGCTAAGACGTTGGGCAATTGTG			299
	A V K L F K F S R E K K A A K T L G I V			
	900	920	940	
898	GTCGGTATGTTTCATCTTGTGCTGGCTACCCCTTCTTCATCGCTCTACCGCTTGGCTCCTTG			957
300	V G M F I L C W L P F F I A L P L G S L			319
	960	980	1000	
958	TTCCTCCACCCCTGAAGCCCCCGACGCCGTGTTCAAGGTGGTGTCTCTGGCTGGGCTACTTC			1017
320	F S T L K P P D A V F K V V F W L G Y F			339
	1020	1040	1060	
1018	AACAGCTGCCTCAACCCCATCATCTACCCATGCTCCAGCAAGGAGTTCAAGCGCGCTTTC			1077
340	N S C L N P I I I Y P C S S K E F K R A F			359

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FIGURE 2G

1318	1320	1340	1360	1377
440	GAGCTGTGCGCCTTCCCGGAGTGGAGCGCGCGCCCTCCTGAGCCTGCCCGGCCT			459
	E L C A F P E W K A P G A L L S L P A P			
1378	1380	1400	1420	1437
460	GAGCCCCCGCGCGCGCCGACGACTCGGGCGCGCTCTTCACTTCAAGCTCCTG			479
	E P P G R R G R H D S G P L F T F K L L			
1438	1440	1460	1480	1497
480	ACGAGCCCGAGAGCCCGGACCGACGGCGCGCCAGCAACGAGGCTGCGAGCCGCG			499
	T E P E S P G T D G G A S N G G C E A A			
1498	1500	1520	1540	1557
500	GCCGACGTGGCCAAACGGGCAGCCGGGCTTCAAAAGCAACATGCCCTGGCGCCCGGCAG			519
	A D V A N G Q P G G F K S N M P L A P G Q			

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1615
520

1600
AAAACATCGTGGGGGGA

1558
520

FIGURE 2H

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FIGURE 3A, 18/37
FIGURE 3B, 19/37
FIGURE 3C, 20/37
FIGURE 3D, 21/37
FIGURE 3E, 22/37
FIGURE 3F, 23/37
FIGURE 3G, 24/37

FIGURE 3A

-120	-100	-80	
-124	CCAGCCAAACCACTGGCAGGCTCCCTCCAGCCGAGACCTTTATTATCCCGGCTCCCGAGCT		-65
-60	-40	-20	
-64	CCGCCCTCCGCGCCAGCCCGGAGGTTGGCCCTGACAGCCGGACCTCGCCCGGCCCGGCTG		-5
0	20	40	
-4	GGACCATGGTGTCTCTCGGGAATGCTTCCGACAGCTCCAACTGCACCCCAACCGCCGG		55
0	M V F L S G N A S D S S N C T Q P P A		19
60	80	100	
56	CACCGGTGAACATTTCCAAGGCCATTTCTGCTCGGGGTGATCTTGGGGGGCCTCATCTTT		115
20	P V N I S K A I L L G V I L G G L I L F		39

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FIGURE 3B

116	120	140	160	175
40	TCGGGGTGGGTAACATCCTAGTATCCTCTCCGTAGCCTGTCA			59
	G V L G N I L V I L S V A C H R H L H S			
176	180	200	220	235
60	CAGTCACGCACTACTACATCGTCAACCTGGCGGTGGCCGACCTCCTGCTCACCTCCACGG			79
	V T H Y Y I V N L A V A D L L L T S T V			
236	240	260	280	295
80	TGCTGCCCTTCTCCGCCCATCTTCGAGGTCCTAGGCTACTGGGCCTTCGGCAGGGTCTTCT			99
	L P F S A I F E V L G Y W A F G R V F C			
296	300	320	340	355
100	GCAACATCTGGGCGGCAGTGGATGTGTGTGTGCTGCACCGCGTCCATCATGGGCCCTCTGCA			119
	N I W A A V D V L C C T A S I M G L C I			

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FIGURE 3C

356	TCATCTCCATCGACCGCTACATCGGCGTGAGCTACCCGCTGGGCTACCCAACCATCGTCA	360	380	400	415
120	I S I D R Y I G V S Y P L R Y P T I V T				139
	420	440	460		
416	CCAGAGGAGGGTCTCATGGCTCTGCTCTGCGTCTGGGCACTCTCCCTGGTCATATCCA				475
140	Q R R G L M A L L C V W A L S L V I S I				159
	480	500	520		
476	TTGGACCCCTGTTCGGCTGGAGGCAGCCGGCCCGGAGGACGAGACCATCTGCCAGATCA				535
160	G P L F G W R Q P A P E D E T I C Q I N				179
	540	560	580		
536	ACGAGGAGCCGGGCTACGTGCTCTTCTCAGCGCTGGGCTCCTTCTACCTGCCTCTGGCCA				595
180	E E P G Y V L F S A L G S F Y L P L A I				199

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FIGURE 3D

596	600	620	640	655
200	TCATCCTGGTCA	TGTA	CTGCGCGGTCT	ACGTGGTGGCC
	I L V M Y C R V Y V A K R E S R G L K			219
	660	680	700	
656	AGTCTGGCCTCA	AGACCAAGT	CGGACTCGGAG	CAAGTGACGCT
220	S G L K T D K S D S E Q V T L R I H R K			715
	720	740	760	239
716	AAAACGCCCCG	CAGGAGG	CAGCGGATG	GGCCAGCAAG
240	N A P A G G S G M A S A K T K T H F S V			775
	780	800	820	259
776	TGAGGCTCCTCA	AGTTCTCCCG	GGAGAAAGC	GGCCAAACG
260	R L L K F S R E K A A K T L G I V V G			835
				279

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FIGURE 3E

836	840	860	880	895
280	GCTGCTTCGTCCTCTGCTGGCTGCCTTTTCTTCTTAGTCATGCCCATTTGGGTCTTTCTTCC			299
	C F V L C W L P F F L V M P I G S F P			
	900	920	940	
896	CTGATTTCAAGCCCTCTGAAACAGTTTAAATAAGTATTTTGGCTCGGATATCTAAACA			955
300	D F K P S E T V F K I V F W L G Y L N S			319
	960	980	1000	
956	GCTGCATCAACCCCATCATATACCCATGCTCCAGCCCAAGAGTTCAAAAAGGCCTTTCAGA			1015
320	C I N P I I Y P C S S Q E F K K A F Q N			339
	1020	1040	1060	
1016	ATGTCCTTGAGAAATCCAGTGTCTCTGCAGAAAGCAGTCTTCCAAACATGCCCTGGGCTACA			1075
340	V L R I Q C L C R K Q S S K H A L G Y T			359

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FIGURE 3F

1076	1080	1100	1120	1135
360	CCCTGCACCCGCCCAGCCAGGCGGTGGAAGGCAACAAGGACATGGTGCGCATCCCCG			379
	L H P P S Q A V E G Q H K D M V R I P V			
1136	1140	1160	1180	1195
380	TGGGATCAAGAGAGACCTTCTACAGGATCTCCAAGACGGATGGCGTTTGTGAATGGAAAT			399
	G S R E T F Y R I S K T D G V C E W K F			
1196	1200	1220	1240	1255
400	TTTCTCTTCCATGCCCCCGTGGATCTGCCAGGATTACAGTGTCCAAAGACCAATCCTCCT			419
	F S S M P R G S A R I T V S K D Q S S C			
1256	1260	1280	1300	1315
420	GTACCACAGCCCGGTGAGAAGTAAAGCTTTTTCAGGTCTGCTGTGTAGGGCCCT			439
	T T A R V R S K S F L Q V C C C V G P S			

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FIGURE 3G

1316	1320	1340	1360	1375
440	CAACCCAGCCTTGACAAGAACCATCAAGTTCCAAACCATTAAGGTCCACACCATCTCCC			459
	T P S L D K N H Q V P T I K V H T I S L			
1376	1380	1400	1420	
460	TCAGTGAGAACGGGGAGGAGTCTAGGACAGGAAAGATGCAGAGGAAAGGGAATATCTT			
	S E N G E E V *			
1436	1440	1460	1480	1435
	AGGTACCATAACCCCTGGAGTTCTAGAGGATTCCCTCGACAAGCTTATTCGATCCAGACATG			466
	1500			
1496	ATAGATACATTGATGAGTT	1514		1495

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FIGURE 4A, 25/37
FIGURE 4B, 26/37
FIGURE 4C, 27/37
FIGURE 4D, 28/37

FIGURE 4A

1	human alpha	mtfrdllsvs	fegprpdssa	ggssaggggg	saggaapseg	40
	H318/3 alpha	
	Rat alpha	mtfrdilsvt	fegprssst	ggsgagggag	tvg.....peg	
	Consensus	MTFRD-LS--	FEGPR--SS-	GGG-AGGG-G	--G-----EG	
41	human alpha	pavggvpvg-	ggggg-vga-	sgednrssa.gepgs	80
	H318/3 alpham-	aalrs-mma-	ylsewrtpty	rstemvqrlr	
	Rat alpha	gavggvpvg.-	tggga-vgt-	sgednqsst.gepg	
	Consensus	-----A	-----V--G	-----	-----	
81	human alpha	ag-ggdvngt	-----	-----	-----m-----	120
	H318/3 alpha	me-vqhtstst	-----	-----	-----m-----	
	Rat alpha	aa-sgevnsg	-----	-----	-----t-----	
	Consensus	--A-----	AAVGGLVVSA	QGVGVGVFLA	AFIL-AVAGN	
121	human alpha	-----	-----	-----	-----t-----	160
	H318/3 alpha	-----	-----	-----	-----t-----	
	Rat alpha	-----	-----	-----	-----a-----	
	Consensus	LLVILSVACN	RHLQVTNRYF	IVNLAVADLL	LSA-VLPFSA	

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FIGURE 4B

human	161	-----	-----	-----	200
alpha	-----	-----	-----	-----	-----
alpha	-----	-----	-----	-----	-----
alpha	-----	-----	-----	-----	-----
Consensus	-----	-----	-----	-----	-----
	TMEVLGFWAF	GR-FCDVWAA	VDVLCCTASI	LSLCTISVDR	
human	201	-----	-----	-----	240
alpha	-----	-----	-----	-----	-----
alpha	-----	-----	-----	-----	-----
alpha	-----	-----	-----	-----	-----
Consensus	-----	-----	-----	-----	-----
	YVGVRHSLKY	PAINTERKAA	AILALLW-VA	LVVSVGPLLG	
human	241	-----	-----	-----	280
alpha	-----	-----	-----	-----	-----
alpha	-----	-----	-----	-----	-----
alpha	-----	-----	-----	-----	-----
Consensus	-----	-----	-----	-----	-----
	WKEPVPPDER	FCGITEE-GY	A-FSSVCSFY	LPMVIVVMY	
human	281	-----	-----	-----	320
alpha	-----	-----	-----	-----	-----
alpha	-----	-----	-----	-----	-----
alpha	-----	-----	-----	-----	-----
Consensus	-----	-----	-----	-----	-----
	CRVYVVARST	TRSLEAG-KR	E-GKASEVVL	RIHCRGAAT-	

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FIGURE 4C

human	alpha	321	-d-ah-mr-a	----	360	-----
H318/3	alpha		-d-ah-mr-a	----		-----
Rat	alpha		-k-yp-tq-s	----		-----
Consensus			A-G--G--S-	KGHT-RSSLS		VRLKFSREK KAAKTLAIVV
human	alpha	361	-----	-----	400	-----
H318/3	alpha		-----	-----		-----
Rat	alpha		-----	-----		-----
Consensus			GVFVLCWFPF	FFVLPLGSLF		PQLKPSEGVF KVIFWLGYFN
human	alpha	401	-----	-----	440	-----
H318/3	alpha		-----	-----		-----
Rat	alpha		-----	-----		-----
Consensus			SCVNPLIYPC	SSREFKRAFL		RLLRQCRRR RRR---L---
human	alpha	441	yg..hhw---	...stsgl-q	480	---ap-alt-l
H318/3	alpha		yg..hhw---	...stsgl-q		---ap-alt-l
Rat	alpha		lasldrr--f	rlrpqpsh-s		---cg-grh-
Consensus			-----RA-	-----R-		PG---L---A-

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FIGURE 4D

human	alpha	481	pdpdpeppgt	pem-apv--r	-k..pps afr	ewrllgpfr-	520
H318/3	alpha		pdpdpeppgt	pem-apv--r	-shpapsag	gcwgrsgdp-	
Rat	alpha	gdag	fgl-qsk--llr	ewrllgp1q-	
Consensus			-----	---Q---AS-	R-----	-----R	

human	alpha	521	-ttqlrakvs	slshkiragg	-q-aeaac-q	-seveavslg	560
H318/3	alpha		-scapkspac	rtrspggars	-q-qgraps-q	-wrlcp*...	
Rat	alpha		-ttqlrakvs	slshkirs.g	-r-aetac-l	-seveavsln	
Consensus			P-----	-----	A-R-----A-	R-----SL-	

human	alpha	561	vphevaegat	cqayeladys	nlret di*	588
H318/3	alpha		
Rat	alpha		vpqdgaeavi	cqayepgdys	nlret di*	
Consensus			VP---AE---	CQAYE---DYS	NLRETDI*	

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FIGURE 5A, 29/37
FIGURE 5B, 30/37
FIGURE 5C, 31/37
FIGURE 5D, 32/37

FIGURE 5A

	1	-----	-----	-----	-----	40
Rat	alpha1b	-----	-----	-----	-----	-----
Hamster	alpha1b	-----	-----	-----	-----	-----
Human	alpha1b	-----	-----	-----	-----	-----
Consensus		MNPDLDTGHN	TSAPA-WGEL	K--NFTGPNQ	TSSNSTLPQL	
	41	-----	-----	-----	-----	80
Rat	alpha1b	-----	-----	-----	-----	-----
Hamster	alpha1b	-----	-----	-----	-----	-----
Human	alpha1b	-----	-----	-----	-----	-----
Consensus		D-TRAI SVGL	VLGAFILFAI	VGNILVILSV	ACNRHLRTPT	
	81	-----	-----	-----	-----	120
Rat	alpha1b	-----	-----	-----	-----	-----
Hamster	alpha1b	-----	-----	-----	-----	-----
Human	alpha1b	-----	-----	-----	-----	-----
Consensus		NYFIVNLA-A	DLLLSFTVLP	FSA-LEVLY	WVLGRIFCDI	
	121	-----	-----	-----	-----	160
Rat	alpha1b	-----	-----	-----	-----	-----
Hamster	alpha1b	-----	-----	-----	-----	-----
Human	alpha1b	-----	-----	-----	-----	-----
Consensus		WAAVDVLCCT	ASILSLCAIS	IDRYIGVRS	LQYPTLVTRR	

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FIGURE 5B

	161		200
Rat	-----	-----	-----
alpha1b	-----	-----	-----
Hamster	-----	-----	-----
alpha1b	-----	-----	-----
Human	-----	-----	-----
alpha1b	-----	-----	-----
Consensus	KAILALLSVW VLSTVISGP LLGWKEPAPN DDKECGVTEE		
	201		240
Rat	--C--C--	-----	-----
alpha1b	-----	-----	-----
Hamster	--Y--S--	-----	-----
alpha1b	-----	-----	-----
Human	--Y--S--	-----	-----
alpha1b	-----	-----	-----
Consensus	PF-ALF-SLG SFYIPLAVIL VMYCRVYIVA KRTTKNLEAG		
	241		280
Rat	-----	-----	-----
alpha1b	-----	-----	-----
Hamster	-----	-----	-----
alpha1b	-----	-----	-----
Human	-----	-----	-----
alpha1b	-----	-----	-----
Consensus	VMKEMSNSKE LTLRIHSKNF HEDTSSSTKA KGHNPRSSIA		
	281		320
Rat	-----	-----	-----
alpha1b	-----	-----	-----
Hamster	-----	-----	-----
alpha1b	-----	-----	-----
Human	-----	-----	-----
alpha1b	-----	-----	-----
Consensus	VKLFKFSREK KAAKTLGIVV GMFILCWLPP FIALPLGSLF		

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FIGURE 5C

321	Rat	alpha1b	-----	-----	360
-----	Hamster	alpha1b	-----	-----m	-----m
-----	Human	alpha1b	-----	-----	-----v
STLKPPDAVF	Consensus	KVVFWLGYFN	SCLNPIIYPC	SSKEFKRAF-	
361	Rat	alpha1b	-----	-----	400
-----	Hamster	alpha1b	-----	-----a	-----
-----	Human	alpha1b	-----	-----a	-----
-----	Consensus	RILGCQC--R	--RRRRRRRR	LG-CAYTRP	WTRGGSLERS
401	Rat	alpha1b	-----	-----	440
-----	Hamster	alpha1b	-----	-----	-----
-----	Human	alpha1b	-----	-----	-----
QSRKDSLDDS	Consensus	GSC-SG--RT	LPSASPSPGY	LGRG--PP-E	
441	Rat	alpha1b	-----	-----	480
-----	Hamster	alpha1b	-----	-----	-----
-----	Human	alpha1b	-----	-----	-----
LCA-PEWK--	Consensus	GALLSL--PE	PPGRRGR-DS	GPLFTFKLL-	

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FIGURE 5D

Rat	alpha1b	481	d-----eat	-----dttt	-l-----	-----g--h-	520
Hamster	alpha1b		e-----egd	-----datt	-l-----	-----a--h-	
Human	alpha1b		e-----dgg	-----eaaa	-v-----	-----a--q-	
	Consensus		-PESPGT---	ASNGGC----	D-ANGQPGFK	SNMPL-PG-F	
Rat	alpha1b	521	*				
Hamster	alpha1b		*				
Human	alpha1b		*				
	Consensus		*				

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FIGURE 6A

FIGURE 6A, 33/37
FIGURE 6B, 34/37
FIGURE 6C, 35/37

	1		40
Human	-----	-----q--a--	-----
alpha1c	-----	-----h--p--	-----
Bovine	-----	-----	-----
Consensus	MVFLSGNASD	SSNCT-PP-P	VNISKAILLG VILGGLILFG
	41		80
Human	-----	-----	-----
alpha1c	-----	-----	-----
Bovine	-----	-----	-----
Consensus	VLGNILVILS	VACHRHLSV	THYIVNLAV ADLLTSTVL
	81		120
Human	-----v--	-----i--	-----
alpha1c	-----i--	-----v--	-----
Bovine	PFSAIFE-LG	YWAFGRVFCN	-WAAVDVLCC TASIMGLCII
Consensus			
	121		160
Human	-----	-----r--	-----
alpha1c	-----	-----k--	-----
Bovine	SIDRYIGVSY	PLRYPTIVTQ	-RGLMALLCV WALSLVISIG
Consensus			

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FIGURE 6B

Human	alpha1c	161	-----	-----	-----	200	-----l--a--
Bovine	alpha1c	-----	-----	-----	-----	-----v--t--	-----
Consensus		PLFGWRQPAP	EETICQINE	EPGYVLFSA	GSFY-PL-II		
Human	alpha1c	201	-----	-----	-----	240	-----
Bovine	alpha1c	-----	-----	-----	-----	-----	-----
Consensus		LVMYCRVVV	AKRESRGLKS	GLKTDKSDSE	QVTLRIHRKN		
Human	alpha1c	241	pa-----ma-	--t-----	-----	280	-----
Bovine	alpha1c	--qv-----vt-	--n-----	-----	-----	-----	-----
Consensus		A--GGSG--S	AK-KTHFSVR	LLKFSREKKA	AKTLGIIVGC		
Human	alpha1c	281	-----	-----	-----	320	-----v-----
Bovine	alpha1c	-----	-----	-----	-----	-----	-----a-----
Consensus		FVLCWLPPFL	VMPIGSFFPD	F-PSETVFKI	-FWLGYLNSC		

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FIGURE 6C

Human	alpha1c	321	-----	-----	-----	360
Bovine	alpha1c	-----	-----	-----	-----	-----
Consensus		INPIIYPCSS	QEFKKAQNV	LRIQCL-RKQ	SSKH-LGYTL	
Human	alpha1c	361	-p--qav---	---m-----	-r-----r---	400
Bovine	alpha1c	-a--hvl---	---l-----	-a-----k---	-----f---	
Consensus		H-PS---EGQ	HKD-VRIPVG	S-ETFY-ISK	TDGVCEWK-F	
Human	alpha1c	401	---m-----	i t-sk-q-s--	-----v----	440
Bovine	alpha1c	--l-----	m a-ar-p-a--	-----l----	-----l----	
Consensus		SS-PRGSAR-	-V--D-S-CT	TARVRKSFL	QVCCC-GPST	
Human	alpha1c	441	--ldk---v-	---v-----	-----*	467
Bovine	alpha1c	--hge---i-	---i-----	-----*	-----*	
Consensus		PS---NHQ-P	TIK-HTISLS	ENGEEV*		

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FIGURE 7A

FIGURE 7A

FIGURE 7B, 36/37

FIGURE 7C, 37/37

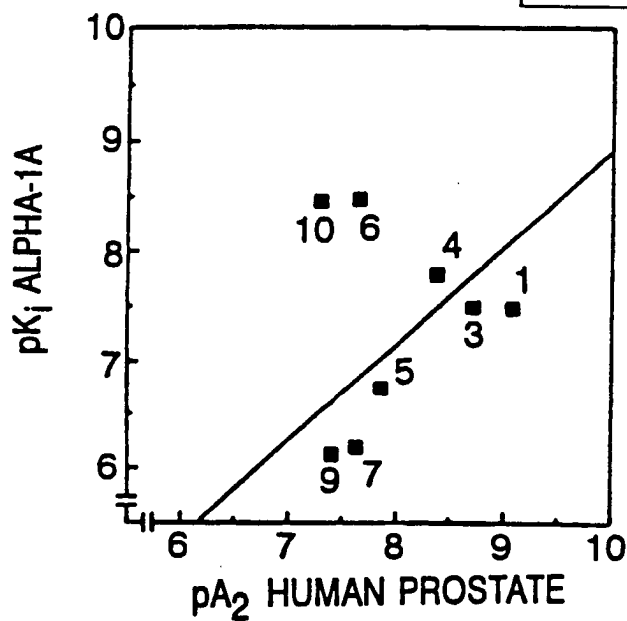
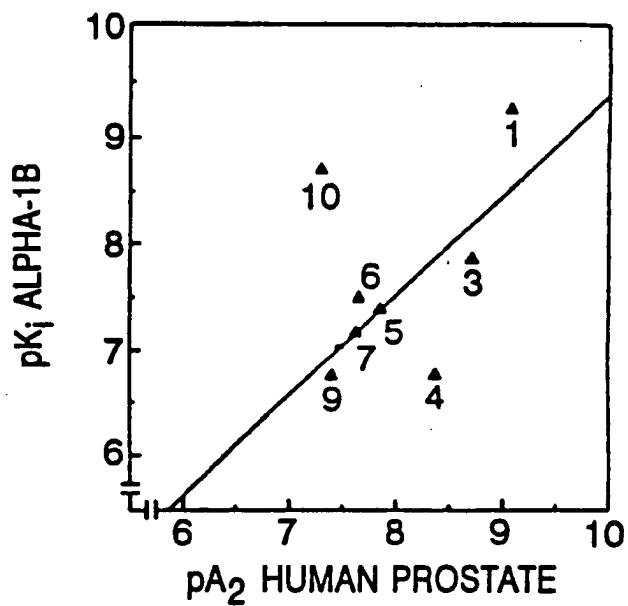
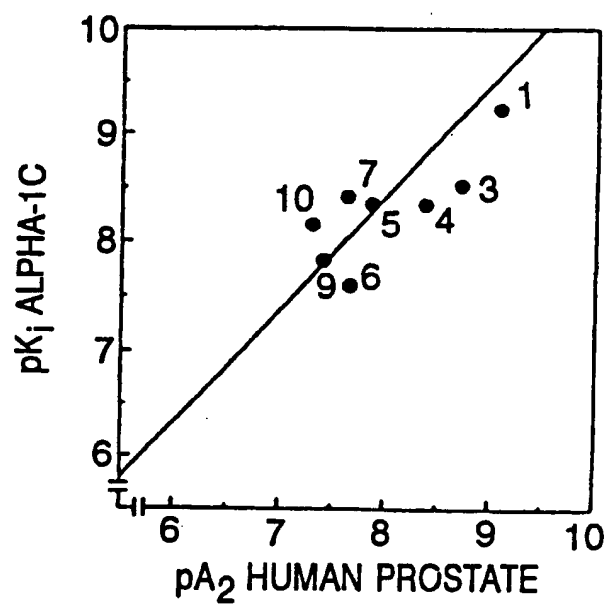


FIGURE 7B



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FIGURE 7C



INTERNATIONAL SEARCH REPORT

International application No.
PCT/US93/09187

A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) :Please See Extra Sheet.

US CL :Please See Extra Sheet.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : Please See Extra Sheet.

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	BIOCH. BIOPHYS. RESEARCH COMM., Vol. 179, No. 3, issued 30 September 1991, Bruno et al., "Molecular Cloning and Sequencing of a cDNA Encoding a Human alpha1a Adrenergic Receptor", pp. 1485-1490, see Fig. 1.	1-142
Y	JOUR. BIOL. CHEM., Vol. 266, No. 10, issued 5 April 1991, Lomasney et al., "Molecular Cloning and Expression of the cDNA for the alpha 1a-Adrenergic Receptor", pp. 6365-6369, see p.6366.	1-142

☒ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* "A"	Special categories of cited documents: document defining the general state of the art which is not considered to be part of particular relevance	"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"E"	earlier document published on or after the international filing date	"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
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"O"	document referring to an oral disclosure, use, exhibition or other means	
"P"	document published prior to the international filing date but later than the priority date claimed	"G" document member of the same patent family

Date of the actual completion of the international search

06 December 1993

Date of mailing of the international search report

JAN 27 1994

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INTERNATIONAL SEARCH REPORT

International application No.

PCT/US93/09187

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	NUCLEIC ACIDS RES., Vol. 18, No. 4, issued 1990, Voigt et al., "Sequence of a rat brain cDNA encoding an alpha-1B adrenergic receptor", p. 1053, see entire document.	1-142
Y	JOUR. BIOL. CHEM., Vol. 265, No. 14, issued 15 May 1990, Schwinn et al., "Molecular Cloning and Expression of the cDNA for a Novel alpha1-Adrenergic Receptor Subtype", pp. 8183-8189, see Fig. 1 and Materials and Methods section.	1-142
Y	EUROPEAN JOURNAL BIOCHEMISTRY, Volume 208, issued 1992, K. Roemer et al., "Concepts and strategies for human gene therapy", pages 211-225, see entire article.	52-76 and 135-137
Y	PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCE, USA, Volume 86, issued September 1989, L.A. Yakubov et al., "Mechanism of oligonucleotide uptake by cells: Involvement of specific receptors?", pages 6454-6458, see entire article.	52-76 and 135-137
Y	THE EMBO JOURNAL, Volume 8, number 12, issued 1989, M. Cotten et al., "Ribozyme mediated destruction of RNA in vivo", pages 3861-3866, see entire article.	52-76 and 135-137
Y	THE JOURNAL OF REPRODUCTIVE MEDICINE, Volume 37, number 6, issued June 1992, E.M. Karson et al., "Prospects for Human Gene Therapy"; pages 508-514, see entire article.	52-76 and 135-137
Y	JOURNAL OF CLINICAL INVESTIGATION, Volume 85, number 4, issued April 1990, L.E. Waspe et al., "The cardiac beta-myosin heavy chain isogene is induced selectively in alpha 1-adrenergic receptor-stimulated hypertrophy of cultured rat heart myocytes", pages 1206-1214, see entire article.	52-76 and 135-137
Y	FASEB JOURNAL, Volume 3, number 8, issued June 1989, G.F. DiBona, "Hypertension and renal alpha adrenergic receptors", pages 1993-1994, see entire article.	52-76 and 135-137
Y	PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCE, USA, Volume 88, issued May 1991, E. Wagner et al., "Transferrin-polycation-DNA complexes: The effect of polycations on the structure of the complex and DNA delivery to cells", pages 4255-4259, see entire article.	52-76 and 135-137
Y	US, A, 4,873,191 (WAGNER ET AL) 10 OCTOBER 1989, see entire document.	52-76 and 135-137

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US93/09187

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US, A, 4,616,017 (BALDWIN ET AL) 07 OCTOBER 1986, see entire document.	52-76 and 135-137
Y	US, A, 4,661,491 (REGNIER) 28 APRIL 1987, see entire document.	52-76 and 135-137
Y	NATURE, Volume 299, issued 14 October 1982, Lerner, "Tapping the immunological repertoire to produce antibodies of predetermined specificity", pages 592-596, see entire document.	47-51 and 117-119
Y	D.M. Glover, "Gene Cloning" published 1984 by Chapman and Hall (London), pages 1-21, see entire document.	33-36 and 38

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US93/09187

A. CLASSIFICATION OF SUBJECT MATTER:
IPC (5):

C12Q 1/00, 1/68; G01N 33/53; C12P 21/06; C12N 5/00, 15/00; A01N 37/18, A61K 37/00; C07K 3/00, 13/00, 15/00, 17/00; C07H 17/00, 19/00, 21/00, 23/00

A. CLASSIFICATION OF SUBJECT MATTER:
US CL :

424/562, 563; 435/6, 7.1, 7.21, 69.1, 240.2, 320.1; 435/172.3; 514/2, 44; 530/350, 387.1; 536/22.1, 23.1, 23.4, 23.5, 24.3, 24.31; 800/2

B. FIELDS SEARCHED
Minimum documentation searched
Classification System: U.S.

424/562, 563; 435/6, 7.1, 7.21, 69.1, 240.2, 320.1; 435/172.3; 514/2, 44; 530/350, 387.1; 536/22.1, 23.1, 23.4, 23.5, 24.3, 24.31; 800/2

B. FIELDS SEARCHED
Electronic data bases consulted (Name of data base and where practicable terms used):

APS, Biosis, Medline, Embase
search terms: human alpha 1 adrenergic receptor, human, adrenergic receptor, alpha 1 or alpha 1, alpha1a, alpha1b, alpha1c, alpha 1a, alpha 1b, alpha 1c, adrenerg?, over (w) express?, overexpress?, hyper?, alpha, beta